The significance of molecular biomarkers in the recurrence of rectal tumors after Transanal Endoscopic Microsurgery

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THE SIGNIFICANCE OF MOLECULAR BIOMARKERS IN THE RECURRENCE OF RECTAL TUMORS AFTER TRANSANAL ENDOSCOPIC MICROSURGERY

MD RESEARCH THESIS DISSERTATION

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Supervisors: Mr Savvas Papagrigoriadis (1st) and Professor Ingvar Bjarnason (2nd)

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To my parents Charalampos and Aikaterini,

my brother Isidoros and sister Christina

who have supported me throughout this journey.

Thesis Declaration

I hereby certify that this MD Research Thesis represents my own work, which was conducted after formal registration in January 2014, within the Transplantation Immunology & Mucosal Biology Research Division, which is part of King's College London. I also certify that all the publications listed in the next page have been prospectively designed and conducted as part of this degree. This MD Research Thesis incorporates publications as per KCL guidelines and policies of "Thesis incorporating publications". Chapters 5, 6, 7, 8 have been published on MEDLINE, and are currently presented unchanged in their final version, as the main body of work of this dissertation.

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Chapter 6: BRAF V600E mutation in colorectal cancer is associated with right-sided tumours and iron deficiency anaemia.

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Chapter 7: KRAS Mutant Status, p16 and β-catenin Expression May Predict Local Recurrence in Patients Who Underwent Transanal Endoscopic Microsurgery (TEMS) for Stage I Rectal Cancer.

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Chapter 8: KRAS Mutant Status May Be Associated with Distant Recurrence in Early-stage Rectal Cancer.

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List of Abbreviations

Abbreviation	Full name
5FU	5 Fluorouracil (chemotherapy)
A/G/C/T	Adenine/Guanine/Cytosine/Thymine
ANOVA	Analysis of Variance
APC	Adenomatous polyposis coli
APR	Abdominoperineal Resection
ASA	American Society of Anaesthesiologists
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
β-catenin	Beta-catenin
BJS	British Journal of Surgery
BMI	Body mass index
BRAF	v-raf murine sarcoma viral oncogene homolog B
BRAF V600E	v-raf murine sarcoma viral oncogene homolog B V600E mutation
CABLE1	CDK5 And ABL1 Enzyme Substrate
CAP	College of American Pathologists
CCD	Charge-Coupled Devices
CEA	Carcinoembryonic Antigen
CIBH	Changes in Bowel Habits
CIMP	CpG island Methylator Phenotype

CIN	Chromosomal Instability
cm	centimetre
COX-2	Cycloxygenase-2
CRC	Colorectal Cancer
CRM	Circumferential Margin
CRT	Chemoradiotherapy
CT	Computer Tomography
c.f. 1799T>A	BRAF V600E mutation
DAB	Diaminobenzidine
DCC	Deleted in Colorectal Carcinoma
DFS	Disease-Free Survival
DNA	Deoxynucleic Acid
EASES	European Association of Endoscopic Surgery
EDTA	Ethylene Diamine Tetraacetic Acid
EGFR	epidermal growth factor
EMVI	Extramural Vascular Invasion
EPR	Electronic Patient Record
ERC	Early Rectal Cancer
ERUS/EUS	Endorectal Ultrasound
ESD	Endoscopic Submucosal Resection
ESMO	European Society of Medical Oncology
EtOH	Ethanol
FAP	Familial Polyposis Syndrome
FFPE	Formalin-Fixed Paraffin-Embedded

FH	Family History
GI	Gastrointestinal
Hb	Haemoglobin
H&E	Hematoxylin & Eosin
HNPCC	Hereditary non-Polyposis syndrome
HR	Hazard Ratios
JCVT	John Cunningham (JC) virus T antigen
JM	Jane Moorhead
IBM	International Business Machines
IDA	Iron-Deficiency Anaemia
IHC	Immunohistochemistry
KCH	King's College Hospital
KRAS	Kirsten rat sarcoma viral oncogene homolog
LAR	Locally Advanced Rectal Cancer
LINE-1 methylation	long interspersed nucleotide element-1 methylation
LN	Lymph nodes
LVI	Lymphovascular Invasion
LOH	Loss of Heterozygosity
LS	Lynch syndrome
MAPK	mitogen-activated protein kinase
MDT	Multidisciplinary Team
Ml	Micro litres
miRNA	micro Ribonucleic Acid

MGMT	06 methylguanine-DNA-methyltransferase
MLH-1	mutL homolog 1
MMR	Mismatch Repair (system)
MSH2	mutS homolog 2
MSH6	mutS homolog 2
MSI	Microsatellite Instability
MSI-H	Microsatellite Instability – High
MSI-L	Microsatellite Instability – Low
MSS	Microsatellite Stable
MRI	Magnetic Resonance Imaging
Mt	mutant
MΩ	Milliohm
(d)NDP	Deoxyribose Nucleoside Diphosphate
NICE	National Institute of Clinical Excellence
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
NSAIDS	Nonsteroidal Anti-inflammatory Drugs
NY	New York
(d)NTP	Deoxyribose Nucleoside Triphosphate
OR	Odds Ratios
P16	cyclin-dependent kinase inhibitor 2A
PCR	Polymerase Chain Reaction
PET	Fludeoxyglucose positron emission tomography

PETACC	Pan-European Trials in Adjuvant Colon Cancer
PHLPP1	PH domain and leucine rich repeat protein phosphatase 1
PHLPP2	PH domain and leucine rich repeat protein phosphatase 2
PI3K/AKT	phosphoinositide-3-kinase / v-akt murine thymoma viral oncogene
PI3KCA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
Pmol	Picomole
PMS2	Post-meiotic segregation increased 2 (S. cerevisiae)
PNI	Perineural Invasion
PPI	Pyrophosphate
RB	Rectal Bleeding
Rca	Rectal Cancer
RFFL	ring finger and FYVE-like domain containing E3 ubiquitin protein ligase
RFS	Relapse-Free Survival
Rpm	Rounds per minute
RNA	Ribonucleic Acid
SDC	Salvador Diaz-Cano
SP	Savvas Papagrigoriadis
SMAD2	Mothers Against Decapentaplegic Homolog 2

SMAD4	Mothers Against Decapentaplegic Homolog 4 (Drosophila)
SRA	Superior Rectal Artery
SPSS	Statistical Package for the Social Sciences
TAR	Transanal Resection
TAMIS	Transanal Minimally Invasive Surgery
TE	
TEMS	Transanal Endoscopic Microsurgery
TME	Total Mesorectal Excision
pTNM (pT, pN, pM)	Pathological Tumour Node Metastasis
UFT	tegafur-uracil oral chemotherapy
USB	Universal Serial Bus
VEGF	Vascular Endothelial Growth Factor
Wnt-pathway	Wingless-related integration site
wt	Wild-type

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1 Summary of the Project

Local excision (LE) of rectal cancer has been practiced as a treatment for 30 years on a highly selected group of patients and tumours. A method of local excision which has recently gained wider acceptance in the treatment of low-grade CRC (T1) is transanal endoscopic microsurgery (TEMS). TEMS offers advantages for operative access and oncological clearance compared to those of transanal resection (TAR). A number of studies have shown that TEMS can have almost equal results to radical surgery for early rectal cancer. Radical surgery has the disadvantage of an approximate mortality rate of 5% and a complication rate of around 20%, and it impacts on quality of life due to stomas and of sexual dysfunction. However, TEMS has a higher local recurrence rate, and efforts have been made to classify risk with morphological and histological criteria.

Molecular biomarkers in the evaluation of CRC prognosis and treatment stratification have been extensively discussed in the literature. Until now, 3 pathways have been identified to explain the background of CRC molecular pathogenesis. Microsatellite instability (MSI) refers to point mutations in the DNA mismatch repair genes. MSI is currently responsible for 15−20% of CRC cases, and there is enough evidence to support its association with prognosis. Chromosomal instability (CIN) is another pathway of carcinogenesis which affects 85% of CRC cases and has been flagged as a poor prognostic marker. CIN encompasses any structural chromosomal abnormality that results in aneuploidy or polyploidy. The third pathway is related to aberrant hypermethylation of suppressor promoter CpG islands, commonly known as CIMP. v-raf murine sarcoma viral oncogene homolog B (BRAF) encodes a serine-threonine protein kinase that acts as a downstream effector of Kirsten rat sarcoma viral oncogene homolog (KRAS) pathways. Various studies have revealed that v-raf murine sarcoma viral oncogene homolog B V600E (BRAF V600E) mutations appear to be valid prognostic indicators. KRAS is a proto-oncogene that encodes a GTPase, which is involved in facilitating cellular response

to extracellular stimuli. KRAS point mutations appear in 40% of CRC cases, and their presence is associated with poor response to anti-epidermal growth factor receptor (anti-EGFR) chemotherapy.

However, to date there has been no literature linking biomarkers with stratification of risk for the treatment of rectal cancer following TEMS.

Aim:

The aim of this dissertation is to assess the significance of these molecular biomarkers in the prediction of local recurrence and prognosis of early rectal cancer following TEMS.

Materials and Methods:

Initially, we performed a narrative review of the literature to consolidate the evidence available for molecular biomarkers in the evaluation of CRC. We then designed a retrospective pilot study to identify the molecular biomarker status of 41 confirmed CRC cases among 1,446 consecutive referrals for suspected cancer. As part of this study, we retrospectively analysed clinical, biochemical, and histopathological data. Gene profile analysis (KRAS, BRAF) of the specimens was also performed.

Following this, we proceeded to analyse data from a series of patients who had undergone TEMS for Stage I rectal cancer at King's College Hospital (KCH). Demographic, biochemical, histopathological, and follow-up data were prospectively collected. Molecular analysis was prospectively performed in the Advanced Diagnostics Laboratory of KCH to identify the status of BRAF, KRAS, p16 O6-methylguanine-DNA methyltransferase (MGMT), and β-catenin.

Finally, we retrospectively collected equivalent data on a 4-year series of 135 confirmed Stage I–IV rectal cancer cases who underwent radical surgery +/- neoadjuvant chemoradiotherapy. Data on the status of the same molecular biomarkers were retrospectively collected. In both

cohorts of rectal cancer cases (TEMS/radical surgery +/- additional treatment), the biomarker status was compared with the histopathology and follow-up outcomes, including recurrence and overall cancer-related survival.

Results:

In our pilot study (41 cases), there was no significant correlation of presenting haemoglobin (Hb) levels with eventual disease staging ($p>0.05$ for all associations). Patients with right-sided tumours were found to have a lower Hb level than patients with either left-sided or rectal tumours. Hb levels were also significantly lower in patients with the BRAF V600E mutation, although this may be because all 3 patients with the mutation had right-sided tumours. Neither KRAS status nor lymphovascular invasion (LVI) status had a specific correlation with Hb levels.

Of 29 specimens of cases who underwent TEMS, there was a statistically significant association between KRAS mutant status and local recurrence (n=6, p=0.037). P16 expression >5% (mean=10.8%, min=0, max=95) was associated with earlier recurrence within 11.70 months (n=7, p=0.004). Membranous β-catenin expression (n=12, 48%) was also related to KRAS mutant (mt) status (p=0.006) but not to survival ($p>0.05$). BRAF gene was found to be wild type in all cases tested (n=23).

With regard to the specimens of rectal cancer cases who underwent radical excision, 28 cases were Stage I (20.9%), n=30; Stage II (22.4%), n=45; Stage III (33.6%) and n=31 Stage IV (23.1. KRAS mt status was associated with female gender (n=20, p=0.021) and older age (69.62 vs. 62.27, p=0.005). Stage I early cancer subgroup analysis showed that KRAS mt status was associated with distant recurrence of disease (n=4, p=0.045).

Conclusions:

BRAF V600E mutation seems to be associated with right-sided CRC and iron-deficiency anaemia. This could be used as an adjunct to diagnostic molecular tests for early diagnosis. KRAS, p16, and β-catenin could be used as biomarkers for prediction of local recurrence and stratification of the risk for further surgery in Stage I rectal cancer. Further to this, KRAS may be a predictor of distant recurrence in cases of early stage rectal cancer.

2 Introduction

2.1 Anatomy of the rectum

The rectum is 15–18 cm in length, anatomically extending from the third sacral vertebra to the dentate line. It can be divided into thirds: the upper intraperitoneal third (covered anteriorly and laterally by peritoneum), the middle third (covered anteriorly by peritoneum), and the lower extraperitoneal third. Anteriorly, Denonvilliers' fascia separates the rectum from the posterior vaginal wall in females, and the bladder, the prostate, and the seminal vesicles in males. Posteriorly, the rectosacral Waldeyer's fascia separates the mesorectum from the sacrum [1].

The mesorectum contains blood vessels, nerves, and lymph nodes, enveloped by the fascia propria of the rectum. The superior rectal artery (SRA), which is a branch of the inferior mesenteric artery, is the main blood supply of the rectum. The SRA is divided into the superior haemorrhoidal arteries. The inferior haemorrhoidal arteries normally arise directly from the internal ileac artery or sometimes from the middle rectal artery; the venous supply follows the arteries. Lymphatic drainage of the rectum is via the inferior mesenteric nodes in the upper two-thirds; the lower third lymphatic drainage can vary along the middle rectal vessels [1].

In this context, local anatomy access for excision of rectal neoplasms can be challenging [2]. Also, the absence of serosa below the peritoneal reflection allows rectal tumours to grow in a deeper anatomic plane. This permits invasion towards the perirectal fat, making any attempt for surgical access a technical challenge.

2.2 Rectal cancer – Is it different from colonic?

Colon and rectal cancer combined (CRC) is the third most common malignancy, with approximately 30% of these tumours arising from the rectum [3]. Its incidence in the UK is 55/100,000 for males and 34/100,000 for females, which has been reduced by almost 10% over

the last 25 years[1]. The relative 5-year survival is 55%, and this is inversely related to increasing age.

Rectal cancer results from a complex sequence of events, which involve a step-by step transformation of the normal rectal epithelium initially to a dysplastic lesion, and subsequently to invasive carcinoma [4]. This process requires the accumulation of either somatic or germline mutations over a period of approximately 10–15 years. The vast majority of CRCs are adenocarcinomas. These can be subdivided further as mucinous, signet ring, and medullary histological subtypes.

The epidemiology, diagnosis, staging, genetic profile, and management plan of rectal cancer differ significantly from the rest of the CRCs. It is difficult to identify the epidemiological features of rectal cancer, as most studies provide information only for combined CRC. In the US, of 13,439 cases of CRC, approximately 39,910 (30%) are due to rectal cancer (Rca); 18% of Rca cases have a young onset, i.e., age <50 years. Low socioeconomic level has a stronger association with Rca than with right-sided CRC [4] .

Accurate staging of Rca is the basis of treatment selection, and this will be discussed later in this thesis. Early stage Rca (cT1N0M0) can be treated with endoscopic resection, whereas upfront resection is applicable when the size of the lesion does not exceed the muscularis propria with negative lymph nodes (cT2N0M0). In the case of locally advanced rectal cancer (cT3–T4N0–2M0), neoadjuvant chemoradiotherapy and radical resection are the gold standard treatments. Adjuvant therapy can be considered in the case of pathological T3 and above, or where there is lymph node involvement [4].

The distinct embryologic and postnatal development of the proximal and distal colon results in a series of different biological characteristics of colonic mucosa; this precipitate different responses to various environmental or carcinogenic factors. Further to this, recent data show that more than 1,000 genes are expressed differently in the adult proximal versus the distal

colon; surprisingly, only 87 genes show a different expression in the fetal ascending versus the descending colon [5]. This indicates that additional gene-regulating processes are also taking place at this time. As we discuss in detail in Chapter 5, the molecular biomarkers of rectal cancer are distinct from those of the rest of the colon. A different embryologic origin, as well as a different pattern of gene expression, can affect the significance of certain genes in the carcinogenesis pathway. The study "A Comprehensive Molecular Characterization of Human Colon and Rectal Cancer" [6], concluded that there are significant differences in hypermethylation trends between the right colon (higher trend) and the proximal colon including the rectum. As discussed extensively in the last chapter, hypermethylation is one of the fundamental events that lead to carcinogenesis.

With regard to survival rates, recent data support that rectal cancer seems to have a similar prognosis to that of colon cancer. A population-based study [7] of 372,130 patients from the Surveillance, Epidemiology and End Results Program database (1995–2008) concluded that rectal and colon cancer had a similar prognosis, based on a 32-month follow-up window.

2.3 Rectal cancer and genes

CRC is generally classified as hereditary and sporadic. The commonest hereditary subtype is familial adenomatous polyposis (FAP). FAP is an autosomal dominant condition that affects 1 in 13,500 members of the general population. FAP is defined as the presence of >100 adenomatous polyps within the large bowel, and there is an almost 100% risk of developing CRC between the third and fifth decades. Lynch syndrome (LS) is another autosomal dominant condition associated with CRC [8]. In LS, there is an increased risk of developing synchronous endometrial or other types of cancers including breast, small bowel, urinary tract, ovarian, and brain cancer. LS is also known as hereditary nonpolyposis colorectal cancer (HNPCC) [8]. Other less common hereditary conditions are juvenile polyposis syndrome, Peutz-Jeghers syndrome, and MYH-associated polyposis.

Sporadic CRC is a multifactorial event in which hereditary, environmental, and other factors are involved [9, 10]. Sporadic CRC is more of a developed country's disease, with North America, Western Europe, New Zealand, and Australia having the highest incidences of occurrence. Lifestyle plays an important role, with obesity and alcohol consumption being directly associated with increased prevalence of CRC. Consumption of 30−45 g/day of alcohol can increase the possibility of developing cancer by 16%, and more than 45 g/day is associated with a 41% increase in risk. The European Prospective Investigation into Cancer and Nutrition (EPIC) has advised that red-meat consumption precipitates a higher risk of CRC, whereas fish consumption is protective for its occurrence. Another possible contributing factor is ulcerative colitis (UC), which is associated with a higher risk of CRC. It has been shown that long-term consumption of nonsteroidal anti-inflammatory drugs (NSAIDS), including aspirin, may reduce the risk of CRC; however, as of yet there is no evidence about the risks and benefits for commencing prophylactic aspirin for CRC.

The progression from normal rectal epithelium to a precancerous lesion and invasive carcinoma is a well-recognized pathway, which results from a step-by-step accumulation of certain mutations [11]. Initially, the transition between normal and mild dysplastic epithelium involves mutations in the APC, bcl-2, and c-myc genes via aberrant hypomethylation of their genes' promoters [11]. Following this, K-ras point mutations trigger a transition from mild to moderate dysplasia, which results in severe dysplasia due to the accumulation of SMAD-2, SMAD 4, DCC, and STAT3 mutations. This process can take up to 20 years (5–20 years), and the lesions that fall into a severe dysplastic genetic profile are still considered to be adenomas. Figure 1 shows the detailed transition from normal epithelium to adenoma and then to carcinoma. P53 gene mutation, p16 aberrant expression, and various chromosomal aneuploidy changes are associated with the step between severe dysplasia and cancer [11].

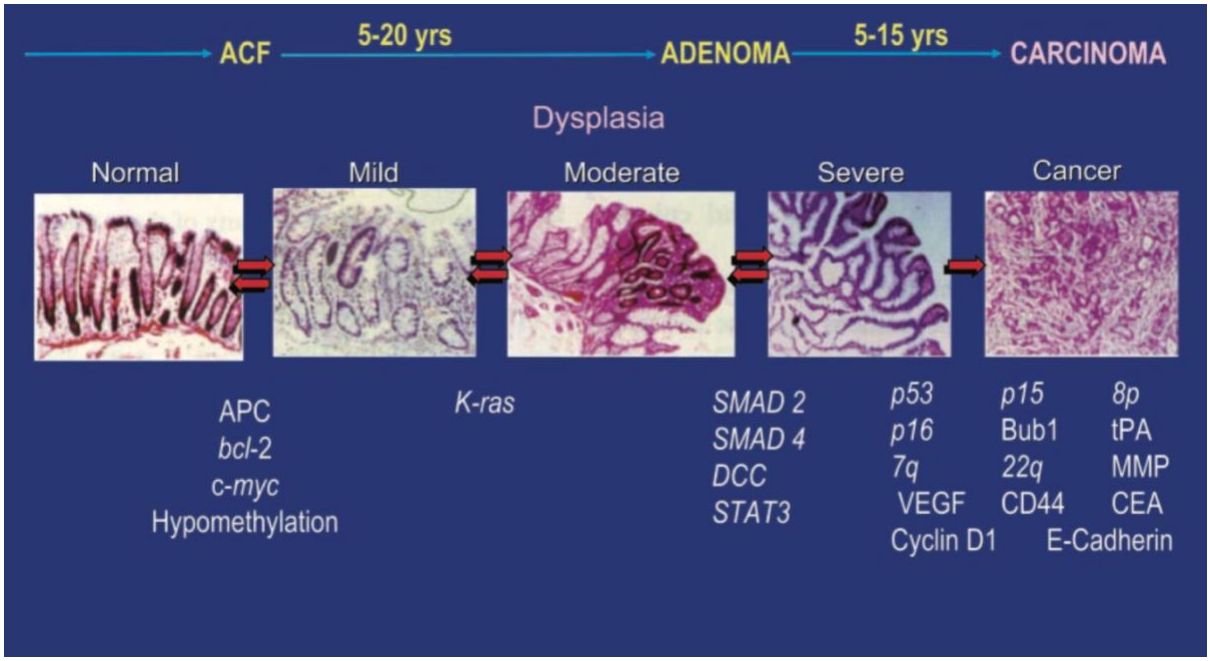

Figure 1. Adenoma–carcinoma transition. (Imported from BJS [11])

2.4 Histopathology

There are several histopathological factors that seem to contribute significantly to the evaluation of rectal cancer. Tumour classification, grade of differentiation, tumour budding, lymphovascular invasion (LVI), perineural invasion (PNI), and the presence of lymph nodes, seem to directly affect prognostic outcomes[3].

According to a recent consensus by the World Health Organization (WHO), CRC is overall divided into certain histologic types, which are summarized in Table 1 [12]; adenocarcinoma is the most common type of CRC.

Types of Colorectal Carcinoma (World Health Organization Classification)
Adenocarcinoma
Medullary carcinoma
Mucinous (colloid) adenocarcinoma (>50% mucinous)

Signet-ring cell carcinoma (>50% signet-ring cells)
Squamous cell (epidermoid) carcinoma
Adenosquamous carcinoma
Small-cell (oat cell) carcinoma
Undifferentiated carcinoma
Other (e.g. papillary carcinoma)

Table 1. Types of colorectal carcinoma (WHO classification) [12]

Tumour differentiation grade has been widely used in the literature as a recognised histopathological prognostic marker; however, interobserver variability has been reported to be an issue. Several systems have been used to grade differentiation [12]; some of them use gland formation as a primary differentiating feature, whilst others employ a more complicated complex of features to determine the grade of differentiation. Either way, most systems define 3 or 4 grades: well-differentiated (grade 1), moderately differentiated (grade 2), poorly differentiated (grade 3), and undifferentiated (grade 4).

The presence of LVI in malignant tissue is considered to be an adverse prognostic feature; this was confirmed by a College of American Pathologists (CAP) consensus statement in 1999 [13]. PNI refers to a complex process in which tumour cells spread along nerve sheaths [14]. Liebig et al [15] concluded that PNI is an independent prognostic marker for cancer-specific and overall disease-free survival; the survival rate was 75% for PNI-negative tumours, versus 25% for PNI-positive. Figure 2 illustrates the different features that pathologists assess for PNI, LVI, and tumour configuration and budding.

As discussed earlier, the mesorectum is a perirectal soft-tissue envelope which contains the superior rectal artery and vein, numerous lymphatics, as well as the rectal branches of the inferior hypogastric plexus. More importantly, since the introduction of total mesorectal

excision in the 1980s [16, 17], pathologists play a key role in the assessment of the mesorectum. This can be used as a surgical performance marker, as well as a way to assess the circumferential radial margin (CRM) [18]. Table 2 shows the grading of the quality of the mesorectum (macroscopic examination)

TME	**Mesorectum**	**Defects**	**Coning**	**Circumferential Radial Margin**
Complete	Intact, smooth	Not deeper than 5 mm	None	Smooth, regular
Nearly complete	Moderate bulk, irregular	No visible muscularis propria	Moderate	Irregular
Incomplete	Little bulk	Down to muscularis propria	Moderate – marked	Irregular

Table 2 Grading of quality and completeness of the mesorectum in a total mesorectal excision. (Imported from [18])

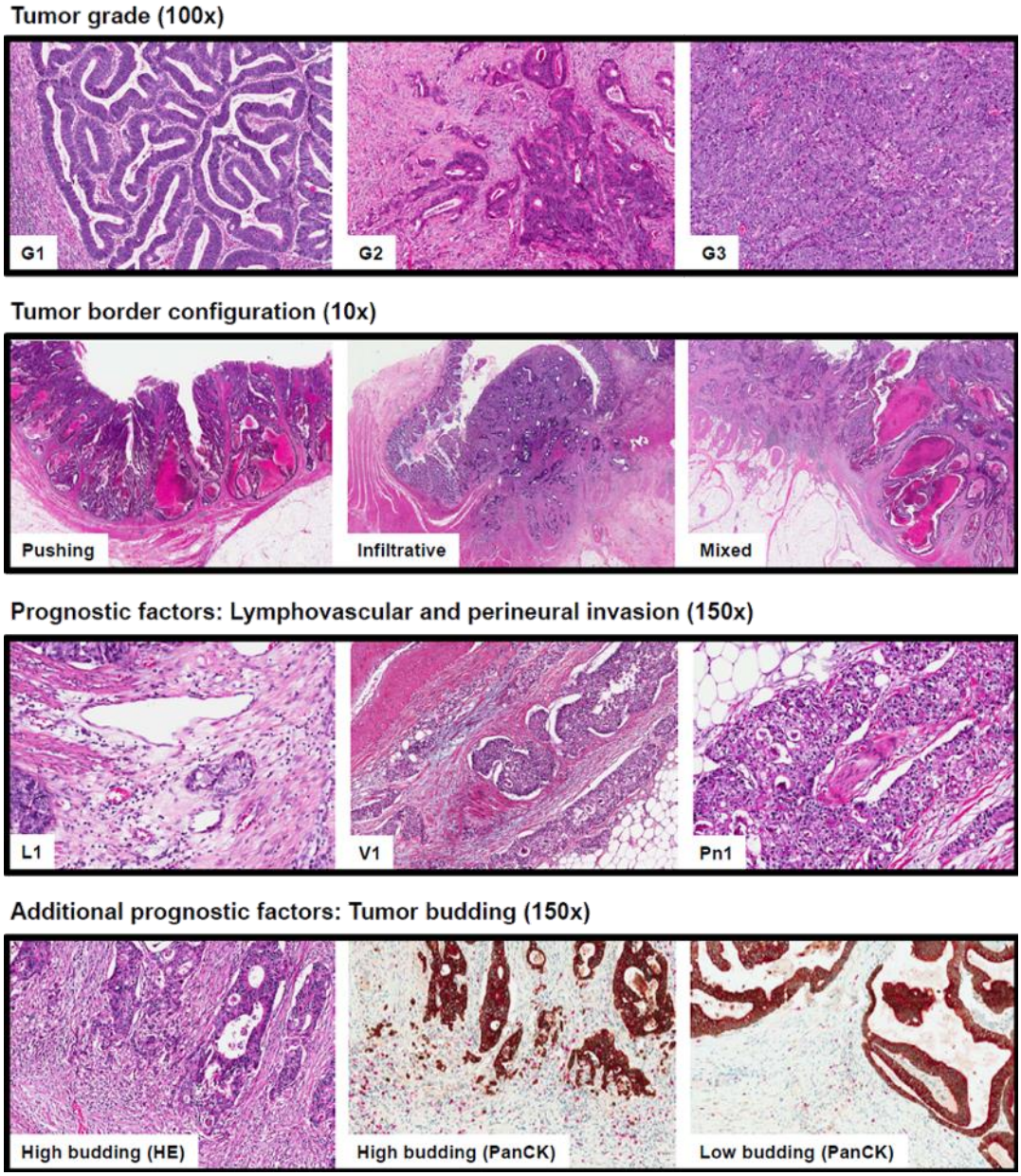

Figure 2: Tumour grade, tumour border configuration, lymphovascular and perineural invasion, and tumour budding, (Imported from http://atlasgeneticsoncology.org/Tumors/colonID5006.html)

2.5 Role of lymph nodes in rectal cancer is unimportant: An argument for MRI evaluation of extramural vascular invasion

Ever since the Dukes era, the presence of lymph node metastasis has been considered an adverse prognostic marker in colorectal cancer. However, this seems to have started to change rapidly. A recent review from Nagtegaal et al. has explained the rationale behind this statement[19], which is based mainly on the fact that most clones which metastasize in liver or lungs are different from those found in LN. This delineates a new perspective for the functionality of LN; basically, this argument converts the function of LN to that of "sponges" rather than "seeds" of cancer cells.

Nagtegaal et al. based their critical reconsideration of LN functionality on a recent study. Naxerova et al. [20] examined the evolutionary relationship between the primary tumour, the lymph nodes, and distant metastases, based on 217 biopsy samples from 17 patients. Naxerova et al. used somatic variants with hypermutable DNA to reconstruct phylogenetic trees (Figure 3). Their conclusion was that in 65% of the cases, distant liver or lung metastases arose from different subclones of primary cancer cells, whereas in only 35% they came from the same clones. Hence, this can be a robust argument for consideration of a different function of LN, primarily as a protective mechanism preventing lateral pelvis spread, rather than as spreading avenues. Based on this theory, measurement of the size of lymph nodes in the MRI preoperative scan would not increase the accuracy of prognosis. Further to this, the presence of large lymph nodes can result in a favourable outcome, especially in the case of stage I/II disease [21]. This strengthens the argument that lymph nodes are involved in the immune system against cancer cells. If this is the case, then which route is the main mechanism for disease spread?

Although vascular spread of rectal carcinoma has been considered for a long time [22], only recently has it begun to be considered as the primary mechanism of rectal disease spread. Initially, cancer cells spread laterally from the pelvic sidewall to the mesorectal margins,

causing local recurrence. Then, they disseminate within the pelvis, entering the peritoneal cavity and metastasizing to the liver via the portal vein. Extramural vascular invasion (EMVI) can be detected by MRI and scored accordingly [23]. In MRI-EMVI–positive cases, the multivariate OR for development of liver metastases is 4.74 (2.06–11.00) [23]. This statement supports the hypothesis of vascular spread mechanism of cancer cells and is also a potential indirect argument for the protective role of LN. On the MRI scan, detection of EMVI post chemoradiotherapy (CRT) can be a more accurate prognostic marker than that of other histopathological parameters [24, 25]. Based on this, downgrading from EMVI-positive to EMVI-negative status post CRT predicts a favourable survival outcome [25], Despite this promising theory, identifying EMVI status on MRI scan or pathology specimens still requires expertise and education to improve sensitivity and specificity.

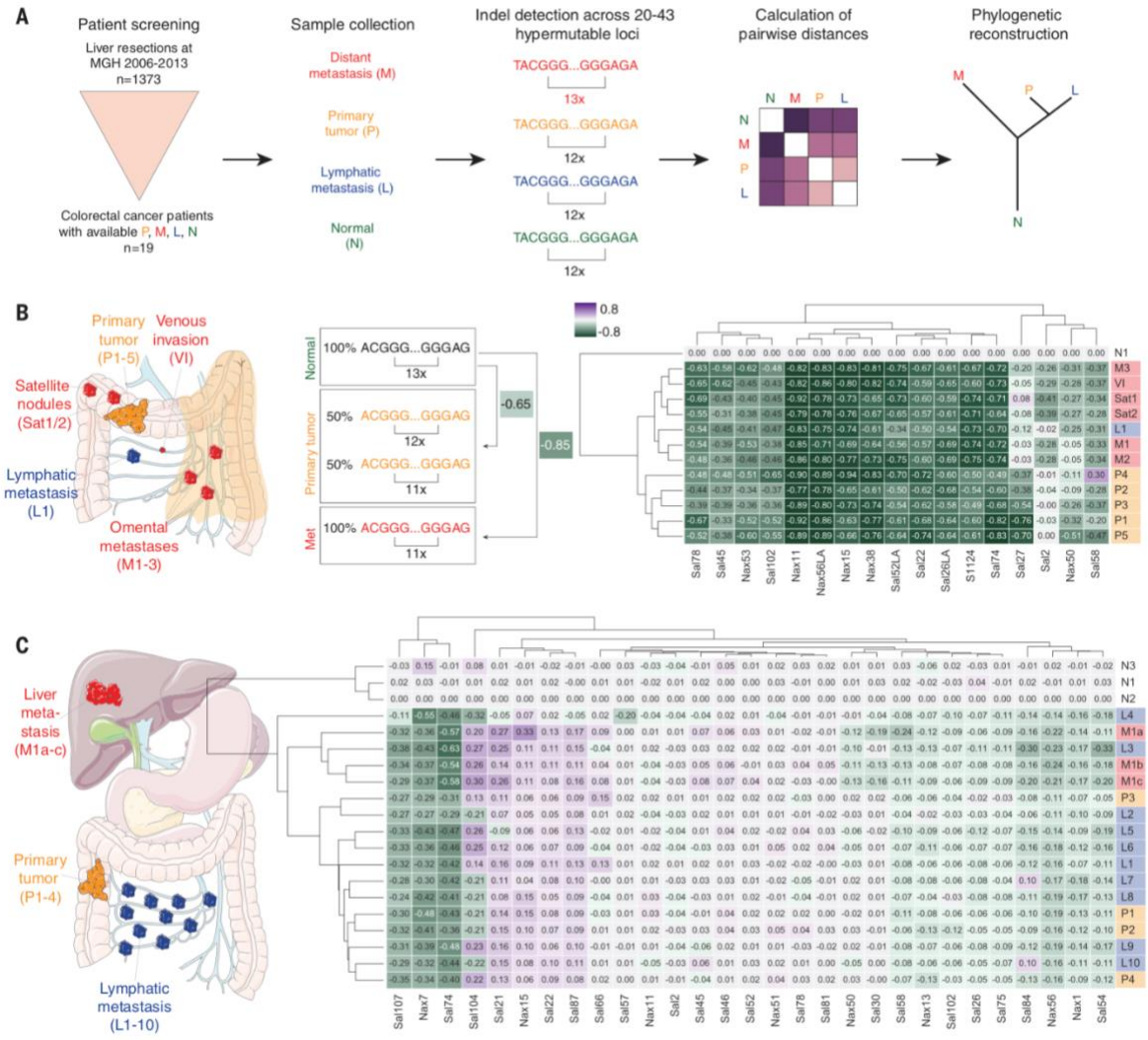

Figure 3. Sixty-five percent of the distant (liver/lungs) and lymph node metastases arose from different subclones of primary cancer cells, whereas only 35% of the metastases arose from the same clone. [20]

2.6 Classification of early rectal cancer

Prior to focusing on the classification of rectal cancer, it is essential to define what we refer to as "early rectal cancer" (ERC). According to the clinical consensus conference of the European Association for Endoscopic Surgery (EASES) [26], ERC is defined as a rectal cancer with good

prognostic features, that can be safely removed with tissue-sparing techniques and can provide good overall survival with limited risk for relapse after surgery.

Tumour node metastasis (TNM) staging is the current gold standard for staging protocols. Although Dukes classification has been used in the past, TNM has gained wider acceptance during the last year. Table 3 shows the TNM and Dukes classification systems, and Table 4 the relevant expected survival rate per stage. For the purposes of this dissertation, we will focus on Stage I rectal cancer. The American Society of Clinical Oncology recommends routine carcinoembryonic serum (CEA) levels and highlights that any increase above 5 ng/ml can be an adverse outcome factor for CRC [27]. Staging computer tomography (CT) of thorax, abdomen, and pelvis is standard practice in most of the cancer units in the UK.

Recently, Kikuchi et al. introduced a new classification for rectal cancer based on the depth of submucosal invasion of the rectal neoplasm [28, 29].Kikuchi classification is widely used in rectal endoscopy. It is based on dividing the submucosa into thirds, and within the uppermost third, there is a separate description of the horizontal spread of the tumour. Rectal neoplasms can be classified from sm1a (less than ¼ of the tumour invading the submucosa) up to sm3 (muscularis propria invasion). Figure 4 illustrates the Kikuchi [29] classification, which is used mainly for early rectal cancer. In a study that included T1 adenocarcinomas, 35% were Sm1, 45% were sm2, and 20% were sm3. Further to this, sm1 lesions have a 3% overall risk for recurrence, while the risk for sm2 recurrent lesions is 8% and 23% for sm3 [7, 8].

	TNM			**Dukes**
Stage 0	Tis	N0	M0	
Stage I	T1	N0	M0	A
Stage II	T2	N0	M0	A
	T3	N0	M0	B
Stage III	Any T	N1	M0	C
	Any T	N2-3	M0	C
Stage IV	Any T	Any N	M1	

Table 3. TNM staging of rectal cancer

UICC	**Disease at the stage of diagnosis**		**5-year survival rate**	
	Colon	Rectum	Colon	Rectum
I	10–12	20	75–100	78–93
II	35–40	25–30	50–60	40–60
III	20–25	20–30	15–40	15–33
IV	18–20	12–20	0–5	0–5
Unknown	6–8	13	-	-

Table 4. UICC 5-year survival rate of rectal cancer

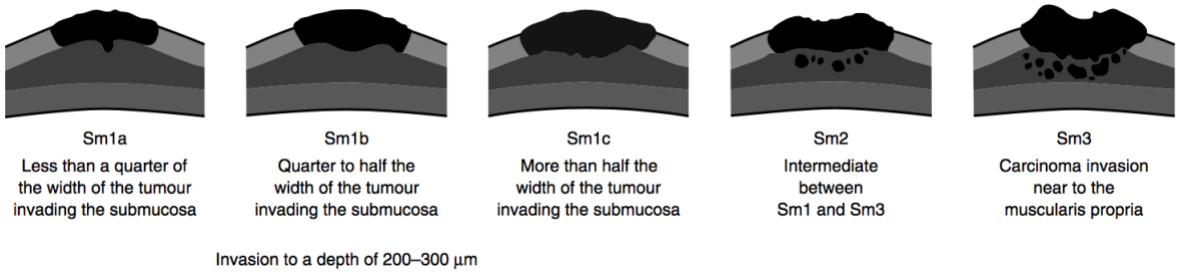

Figure 4. Kikuchi classification (Imported from BJS)[30]

Haggitt et al. [31] described an alternative classification for early rectal cancer, which is less useful for classification of sessile tumours. Figure 5 shows the 4 levels on which Haggitt classification is based. Levels 1–3 refer to pedunculated lesions, whereas by definition any invasive carcinoma should be level 4. Therefore, the major disadvantage of the Haggitt classification is in the case of sessile lesions, for which Kikuchi et al. describe the grade of invasion in detail.

Recently, an international group of surgeons, pathologists, and endoscopists reached a consensus for the endoscopic classification of superficial neoplastic lesions in the oesophagus, stomach, and colon. This classification is named after the city of Paris, where this meeting took place [32]. According to the Paris classification system, there are 2 main macroscopic types of lesions: superficial (type 0) and advanced cancers (types 1–5) [33]. Superficial lesions are further classified based on the distance from the mucosal layer. Those lesions that are elevated >2.5 mm from the mucosal layer are classified as polypoid, while those that are elevated <2.5 mm from the mucosal layer are classified as non-polypoid type lesions; the third category refers to mixed type. Table 5 shows the further subdivision of polypoid, non-polypoid, and mixed types of type 0 lesions according to the Paris classification.

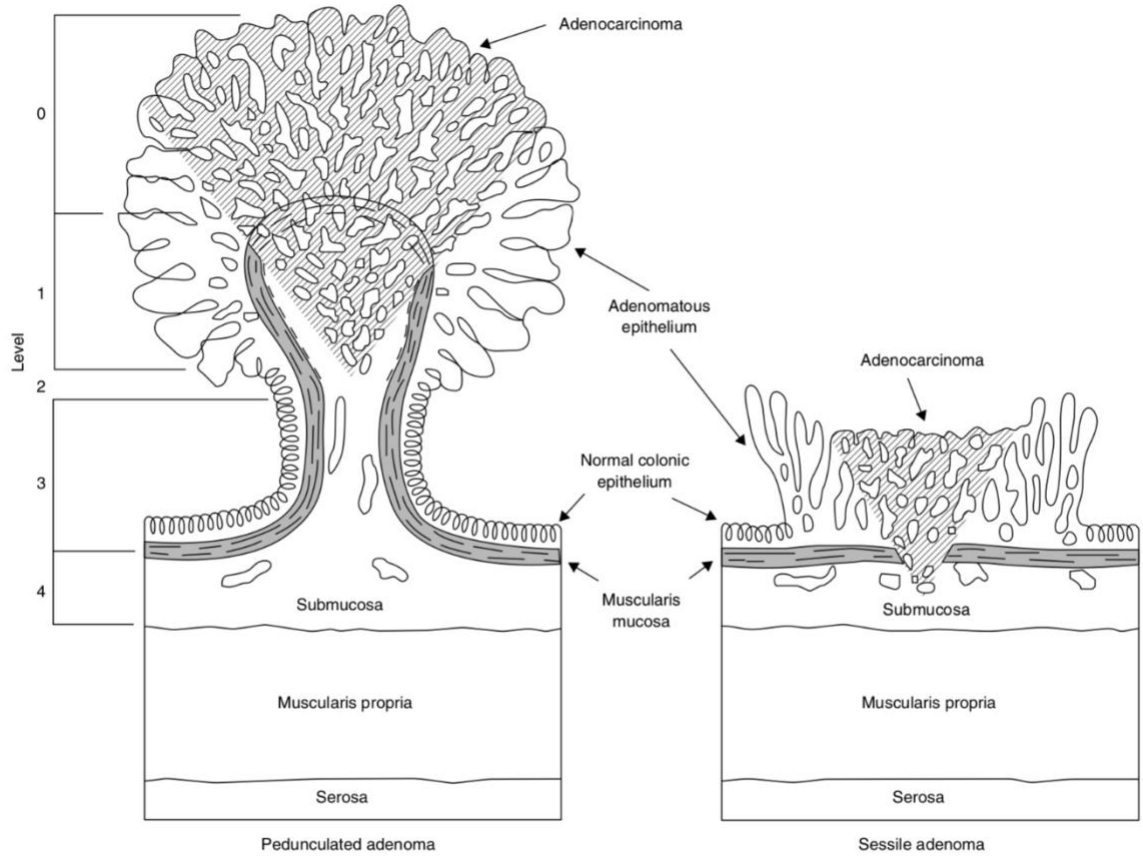

Figure 5. Haggitt classification. (Imported from [30, 31])

Polypoid/Non-Polypoid Type	**Paris Classification**
Polypoid type	Pedunculated (0–1p)
	Sessile (0–1s)
	Mixed (0–1sp)
Non-polypoid type	Slightly elevated (0–IIa)
	Flat (0-IIb)
	Slightly depressed (0-IIc)
Mixed types	Elevated and depressed (0-IIa + IIc)
	Depressed and elevated (0–IIc + IIa)
	Sessile and depressed (0–1s + IIc)

Table 5 Paris classification of superficial colorectal lesions. (Imported from [33])

2.7 Rectal cancer surgery

2.7.1 Historical evolution of radical surgery techniques

Current treatment options for rectal cancer include total mesorectal excision (TME) and transanal excision techniques, which primarily refer to local resection techniques; we will discuss these later in this chapter.

For many years, TME has been accepted as the gold standard treatment option in cases of locally advanced rectal cancer (LAR). Abdominoperineal resection (APR), the surgical treatment of rectal cancer, has been marked by the concept of total mesorectal excision (TME), introduced by Heald et al. [16, 34]. Historically, back in the 19th century, Jacques Lisfranc was the first to describe a transanal approach to remove a rectal tumour (1839). Following this, in 1885, Kraske et al. managed to remove a rectal tumour using a transsacral approach. By that time, colostomy was the cornerstone for treatment of rectal cancer, and symptomatic relief was one of the treatment's main goals and outcomes. Later, in 1908, Ernest Miles described APR. The development of that (at the time) innovative technique was due to the high recurrence rate of rectal cancer, primarily attributed to the extended preservation of tissue, i.e., muscles or lymph nodes. Ernest Miles preferred to proceed with radical surgery of the anorectum, and the oncological outcomes were promising [35]. Despite its oncological superiority, APR holds a high rate of morbidity and mortality, as it is a major procedure which in many cases requires the use of blood products and high dependency support in the postoperative period. More specifically, the overall complication rate can be up to 30%; the mortality rate in the UK based on a recent audit is up to 4.5%. Further to this, APR can result in sexual dysfunction, the need for a temporary or permanent stoma, and loss of social autonomy.

Following this novel approach, Dixon et al. introduced a new technique called anterior resection, which initially was implemented only for upper rectal tumours. However, due to the rapidly evolving technology, this technique was slightly modified for the treatment of lower

rectal lesions as well. This was primarily attributed to the development of specially designed stapling devices through which anorectal and even colorectal anastomosis became possible [35].

2.7.2 Challenges in rectal cancer treatment: TME

As discussed previously, TME was introduced in the late 1880s and early 1890s, when Heald [16] was recognised as the father of this breakthrough in rectal surgery. TME was the first technique to allow low anastomosis with an acceptable rate of local recurrence of disease. Following the Report of the Tripartite Consensus Conference on Definitions for Anorectal Physiology and Rectal Cancer, Washington, DC, in 1999, TME was defined as complete mesorectal excision to the level of the levators [36]. Although it has evolved since then and has been modified several times, the principle of TME is based on Moynihan's observation in 1908 [37] that the mesorectum is involved in lymphatic spread. Heald et al. [17] highlighted the important role of the mesorectum in cancer dissemination until the terminal stages.

TME has been established as the gold standard in the case of middle and lower rectal tumours as it is thought to offer lower recurrence rates of around 6.6%, based on a 5,000-case series [37]. This evidence is primarily derived from a series of retrospective studies since a randomized controlled trial would be difficult to justify in terms of ethical approval.

However, TME is associated with a series of adverse effects. The incidence of anastomotic leaks is estimated at around 20%, although this can be reduced given the better experience that surgeons have these days with TME [37]. A solution to anastomotic leaks could be a routine synchronous diversion, which tends to be performed more in cases of male pelvis. TME has also been associated with gastrointestinal dysfunction, i.e., incontinence; this can be limited to 20% with nerve-sparing approach techniques, which are practiced by the vast majority of surgeons [37]. Finally, in some cases urinary and sexual dysfunction has been attributed to TME, although evidence for this is still inconclusive. Enker et al. [38] supported the fact that

sexual functionality can be maintained in up to 80% of the cases, depending on patient age and surgical approach.

2.7.3 Local excision options for early rectal cancer

The definition of ERC has been discussed previously. Local excision (LE) of rectal tumours has been practiced globally for more than 30 years [39] [40], which follows the overall concept of a tissue-sparing surgical choice, if possible.

The choice between radical surgery and local excision has been extensively discussed in the literature, although it can still be controversial. Currently, treatment stratification is facilitated in an MDT environment. Improved staging techniques including CT, MRI, and high-frequency ERUS have facilitated more accurate staging and potentially better treatment options. Optimal staging techniques are truly important, as they allow better prediction of ERC prognosis and hence, optimal treatment stratification (local versus radical excision). LE is a broader term encompassing several different surgical techniques, which reflects the relevant evolutionary process. LE is considered as a safe alternative in the case of ERC. Transanal excision (TAR) is an example of an older local resection option which has been practiced for some time [41]. Traditional full-thickness TAR is limited to tumours <4 cm in diameter that are within 5 cm [41] from the anal verge; this has recently been extended to 6–8 cm from the anal verge [42]. Access to lesions is facilitated by anal retractors, and local anaesthetic infiltration helps in minimizing blood loss intraoperatively. The main disadvantage of TAR is that it is suitable for excision of only a very small number of lower rectum adenomas.

First described in 2010, transanal minimally invasive surgery (TAMIS) [43, 44] is practiced in several hospitals across the world. The TAMIS surgical platform, which is based on a single-port transanal platform combined with the usual laparoscopic equipment, is designed to bring

access to proximal rectal tumours. In the US, 2 FDA-approved devices have been used: the Gel-POINT and the SILS Port [44]. As with every other LE technique, TAMIS has strict patient selection criteria, which in most cases are limited to well-differentiated adenocarcinomas, pT1 stage without PNI or LVI, and size <4 cm.

Transanal endoscopic microsurgery (TEMS) is a widely used local excision technique that seems to be gaining more and more ground in the treatment of early rectal cancer. TEMS is described in detail in a later chapter. For the scope of this thesis, we based our observations on patients who underwent TEMS.

2.7.4 LE advantages and disadvantages

There are several advantages in favor of LE techniques. A recent meta-analysis [45] concludes that LE offers a better quality of life with fewer postoperative complications. According to this study, the perioperative mortality RR is 0.31 (0.14–0.71); the major post-operative complication RR is 0.20 (0.10–0.41); and the need for a permanent stoma RR is 0.17 (0.09–0.30). In total, this supports a better quality of life following LE.

The main disadvantage of LE is a higher rate of local recurrence; in general, most of the prospective and retrospective studies across the literature report higher recurrence rates associated with LE. According to a recent meta-analysis, the RR is 1.90 (0.57–6.32), which overlaps the line of no effect [46]. However, in a meta-analysis by Kidane et al. [45], which included 2,855 patients in 12 observational studies and 1 randomized controlled trial, a significantly higher mortality rate (72 more deaths per 1,000 patients, CI: 30–120) was reported. A sub-group analysis focused on patients who underwent TEMS yielded a similar mortality rate with radical resection. In the same meta-analysis, the RR for overall recurrence following LE was 2.23 (1.52–3.25); this was based on 1,062 patients who underwent LE versus 1,399 patients who had radical excision. The RR for local recurrence after 5 years based on 13 observational studies varied between 1.48–36.56 [45].

Finally, many authors stress patient selection as a vital parameter in LE practice, and most of the studies consider LE as an option only in cases of early cancer, or for patients unfit for major surgery. With regard to TEMS as a LE option, in cases of early rectal cancer it offers similar oncological outcomes with less morbidity and mortality[41], which we will discuss in detail in the next section.

On the other hand, TME generally runs the risk of development of intraoperative and postoperative complications, with an expected mortality of approximately 2−3% [47]. There have been several references in the literature which conclude that the complication rate of radical surgery is estimated to be around 20−30%. Complications include sexual impotence, decreased fecundity in females, as well as change in bowel habits and the possibility of the need for a permanent stoma, which were discussed previously [48].

2.7.5 Transanal endoscopic microsurgery (TEMS) – Technique and advantages

The method of local excision that has recently gained wider acceptance for early CRC is TEMS, which generally offers advantages for operative access and oncological clearance compared to those of TAR [41].

TEMS is a minimally invasive technique which was introduced by Buess in the early 1980s [49]. With the use of the novel (for that time) rectoscope, which offers 3D binocular optic, and the use of laparoscopic instruments, TEMS offers better access to proximal lesions with lower margin positivity and fragmentation, as well as magnification of the operative field [50, 51]. One of the main advantages of this technique is the fact that it does not impair anorectal function [52]. Multiple studies suggest that TEMS is the gold standard technique for rectal adenomas [50, 51] and retrorectal and submucosal rectal lesions [53]. Enthusiasts like the Italian team of Lezoche et al. [54] published one of the first randomised trials of TEMS vs.

TME for T1−T3 rectal tumours (negative lymph nodes) with additional neoadjuvant CRT [54]. The results were impressive: the local recurrence rate for TEMS was 5.8% vs. 2.8% for TME, and both techniques had 1 distant recurrence each (2.8%).

As discussed previously, radical surgery has the disadvantage of a mortality rate of around 5% and a complication rate of around 20%, with an effect on quality of life from stomas and sexual dysfunction [55] [56, 57]. TEMS is increasingly being supported as the gold standard in early stage rectal cancer [58] and is suitable for surgical treatment of broad-based adenomas [59-61]. In a retrospective study, Christoforidis et al. reviewed information on pT1 and pT2 rectal adenocarcinomas from 1997 through mid-2006. This study included 42 TEMS and 129 TAE patients and concluded that TEMS offers a better quality of resection compared to TAE [62].

2.8 TEMS – Indications and patient selection

During the last decade, a new era of research has evolved which has essentially converted TEMS into a legitimate option for selected patients with early rectal cancer, regardless of the operative risk. The National Institute for Health and Care Excellence (NICE) has incorporated TEMS as a treatment option in such cases [63].

TEMS is an accepted method provided there are no positive lymph nodes present (TNM classification N0). TEMS is indicated as a curative treatment for malignant neoplasms that are histologically confirmed as pT1, sm1 carcinomas. On the other hand, TEMS treatment for T1, sm2–3, and T2 lesions is still controversial [56]. A prospective study by Puia et al. suggests that TEMS is a safe technique for benign early rectal tumours and selected malignant neoplasms [64]. With regard to the previous data, another retrospective study[65] concludes that TEMS is an effective minimally invasive technique for early neoplasms, and when compared to TAR, it has a broader operative range and a better therapeutic effect.

In the case of T3 tumours treated with TEMS, there remains an unacceptably high recurrence rate of approximately 50%. T1N0M0 tumours are currently the gold standard indicators, and

the reported recurrence rate in the literature varies from 5−12%. According to the ESMO guidelines [66], because T2N0M0 tumours are confined within the rectal wall, they can also be candidates for TEMS.

Potentially adverse histological features, as well as the fitness and perioperative risk of the patient are factors taken into consideration. Close follow-up of patients is crucial for early recognition of recurrence, as well as for improvement of their survival outcomes.

Either way, strict patient selection, close follow-up, and the surgeon's level of experience are vital factors that determine TEMS outcomes.

2.9 Oncological outcomes of TEMS

Studies across the literature support the superior oncological outcomes of TEMS compared to those of other local resection surgical techniques [62]. Sajid et al [47] in their meta-analysis, included all published trials to compare the outcomes (morbidity and recurrence) of TEMS versus radical resection for ERC. There was a trend toward higher risk of local recurrence [OR 2.78 (1.42-5.44)] and overall recurrence ($p<0.001$). However, the risk of overall survival and mortality [OR 0.90 (0.49-1.66)] was similar for both TEMS and radical resection. As a limitation for this meta-analysis was reported the significant variation in the design of the included trials.

Studies which contained more than 10% of pT3 tumours and provided neo+/-adjuvant treatment in more than 35% of cases, had improved OR for survival [32.2 (95%CI=16.3-63.5, p=0.001, Q=8.4, p=0.21)]. Studies with more than 10% of pT3 tumours and neo+/-adjuvant treatment in more than 20% of the cases had recurrence-free benefit [OR 20.23 (95%CI=13.84-29.57, p=0.000, Q=2.18, p=0.54)] [67].

A big dilemma in LE is completion surgery in the case of non-complete excision. Hahnloser et al [68] in their study examined the impact of completion surgery within a month. 52 T1N0 or T1N1 rectal cancers were excised locally followed by radical surgery and TME. Further surgery was decided on the basis of unexpected cancer, high risk histology, positive margins, or T3 stage. 29% showed residual cancer in specimen. 21% (mostly in lower rectum) had positive lymph nodes. In conclusion this study showed similar local recurrence after completion TME compared to primary radical resection with TME.

In conclusion, TEMS is associated with higher recurrence rate for T1 and T2; compared though with other LE techniques, it has better oncological outcomes. However, no literature to date has linked biomarkers with risk stratification for the treatment of rectal cancer with TEMS.

2.10 Molecular prognostic and predictive biomarkers – Where are we now?

Currently, treatment strategies and decision-making in the treatment of rectal cancer are primarily based on the latest TNM classification. The rationale behind treatment stratification includes local tumour invasion, spread to lymph nodes or other organs, and is further characterised by the histology (grade of tumour, LVI, differentiation, etc.). Although TNM seems to be the cornerstone for the way rectal cancer treatment is perceived, new data from molecular biology have generated hope and demand for a new era. In general, a biomarker is defined as an objectively measurable characteristic which can describe progression of a biological process or pharmacological response to treatment [69, 70]. Biomarkers are further divided into prognostic or predictive. Prognostic biomarkers refer to measurable characteristics that predict survival, whereas predictive biomarkers focus on response to pharmacological treatment [71, 72]. The identification of molecular biomarkers in the management of CRC has been extensively studied in the literature [73-79]. Researchers have focused on developing classification models based on those biomarkers in order to direct CRC management on the

basis of molecular events. These classification models [80-82] are based on grouping molecular events based on common features which could potentially define certain pathways of carcinogenesis. The starting point in most cases is the progression from healthy epithelium to adenoma and then to carcinoma, which happens in a sequence of events [83, 84].

The current molecular classification models divide CRC into 3 main mechanisms of carcinogenesis. CIN is thought to be involved in 80−85% of CRC and simply refers to any structural abnormality of the chromosomes that can cause aneuploidy and lead to cancer development [73]. MSI affects 10−20% [73, 78] of cases of CRC. The mechanism behind MSI is mismatch defects on the gene repair system, which normally affects repetitive, small DNA sequences called microsatellites. In the vast majority of cases, MSI and CIN cannot coexist together, as this would lead to lethal accumulation of mutations. CIMP is the hypermethylation of CpG islands in various oncogenes or regions of tumour suppressor promoters, which causes upregulation of the latter and therefore cancer progression. CIMP can interfere and overlap with either CIN or CIMP [73, 78]. The prevalence of CIMP is around 20%, which fluctuates depending on the population [73]. CIN, MSI, CIMP tumours or the combinations discussed above can share common characteristics, which are extensively discussed in the last section of this dissertation. In general, CIN is associated with a poor prognosis, whereas MSI-positive tumours have a more favourable prognosis [73]. MSI features include mucinous and poor differentiation, proximal location, female gender, and greater overall 5-year survival. CIMP and CIN combined tumours are distally located, whereas CIMP and MSI combined are located more proximally.

Other important biomarkers in CRC involve the status of KRAS and BRAF genes. KRAS is a proto-oncogene involved in cellular response to various extracellular stimuli. As discussed at length in several chapters of this dissertation, KRAS mutations lead to structural activation of downstream signalling pathways, including mitogen-activated protein kinase (MAPK) and

phosphoinositide-3-kinase/v-Akt murine thymoma viral oncogene (PI3K/AKT) [78, 85]. KRAS status is a well-established CRC molecular biomarker, and most of the literature up until early 2010 concluded that KRAS wild type status can predict favourable response to anti-EGFR inhibitors [76, 85]. However, newer evidence associates KRAS status with prognostic parameters, especially in cases of metastatic or advanced-stage CRC [86-92]. BRAF plays a similar role in intercellular signalling by encoding a serine-threonine kinase that acts as a downstream activator of MAPK pathways [82, 93, 94]. BRAF is thought to be a poor prognostic marker which is primarily found mutated in cases of proximal tumours [93] and can be associated with MSI status. The most common mutation is BRAF V600E, which has also been extensively discussed in cases of skin cancer. More recently, references in the literature discuss a predictive role of BRAF in anti-EGFR response to chemotherapy [95], claiming that BRAF V600E mutation can predict poor response to chemotherapy, regardless of KRAS maintaining wild type status. A recent meta-analysis concluded that BRAF V600E can supplement standard clinical and pathological staging for more individualised management of CRC [93].

On performing a review of the literature [78], we identified biomarkers that are thought to be involved in the molecular classification of CRC. Although there have been several high-quality studies, the complexity and diversity in molecular events have led to limited consensus of their role, including their prognostic or predictive value. Moreover, the initial objective to define a classification model is still in progress, and a recent systematic review [96] concluded that more research is needed to achieve this goal. Consequently, based on the literature evidence we focused our research on those biomarkers that seem to be important in CRC prognosis and framed our questions on outcomes that have not been previously associated with those biomarkers. For instance, there is very limited evidence on the association with biomarkers with recurrence, as well as their association with several histopathological features including

tumour mass, LVI, and PNI. Moreover, looking back to the embryologic origin of rectum, the proximal and distal colon tend to have different biological behaviours, suggesting the potential for different biomarkers. This is why most of the biomarkers tend to be positive (or mutated) in the right colon (i.e. BRAF V600E mutation) but not in the rectum. Looking at the literature, there have been many studies that have investigated the different gene expressions in cancers of the left colon, including the rectum and the right colon [5, 97, 98].

In the context of identifying several biomarkers that have been studied in CRC and identifying which of those can be important to rectal cancer, we have to incorporate an additional challenge. Most of these biomarkers have been studied in more advanced stage (stage II and above CRC. The diagnosis of early rectal cancer is still a controversial topic and is less studied compared to advanced or metastatic CRC. Additionally, newly implemented screening strategies have increased the proportion of cancers that are caught at an early stage. It is important to consider early cancer as a different subgroup that has a complex biological activity, as it can involve several different cancer pathways before advancing to further stages. Tissue-sparing surgery like TEMS in the complex context of early rectal cancer would be effectively optimised if treatment stratification were based on individualised molecular biomarkers. This can provide a paradigm shift in the early prediction of recurrence and the optimisation of treatment options to ensure that high-risk groups are receiving the necessary additional treatment, i.e., neoadjuvant or adjuvant radiotherapy. Based on this statement, oncological outcomes will be equal to those of radical surgery options, whilst achieving dramatically reduced rates of morbidity and mortality. Hence, we focused our research on determining the significance of molecular biomarkers commonly applied in CRC, specifically in the subgroup of early rectal cancer following transanal endoscopic microsurgery.

2.11 Personalised Medicine in colorectal cancer biology: future endeavours

Personalised Medicine is an evolving field which aims to tailor therapy to individualised characteristics of each patient, and, primarily their genetic profile [99]. The general vision of personalised medicine is to offer the right treatment to the right patient at the right time [ref JS]. Treatment of cancer is an eminent example of personalised medicine, and prognostic and/or predictive biomarkers can serve as a fundamental tool for this scope. The hallmarks of cancer as described by Hanahan & Weinberg [100] provide the biological basis explaining why many cancer therapies fail. The wide variation in mutations support the notion of focusing on the individual patients' characteristics in the prevention, diagnosis, prognostic stratification and treatment of cancer; molecular biomarkers have been the stepping stone to achieve this goal.

A classic example of cancer prevention strategy based on genetic features has been inherited from breast cancer biology. Identification of females with BRCA 1 or BRCA 2 mutation and subsequent high risk for future breast cancer development (45-65%, [101]) by the age of 70, has result in establishment led into application of screening programs to identify high risk individuals. Genetic screening for women with relevant risk factors is available in most developed countries' health care services. BRCA 1 and 2 have been associated with high risk for future development of ovarian, colon or prostate cancer. Further to screening protocols, preventive strategies consider removal of breast tissue, or prophylactic oophorectomy or chemical deprivation of oestrogens [99].

In the case of CRC, screening programs have been drastically improved over the last years. As discussed earlier, CRC is a complex event attributed, in most cases to an accumulation of a series of mutations. Around 1% of CRC are caused by FAP which preserves a certain genetic profile; in most cases this affects the *APC* gene which results in down-regulation of wnt

signalling pathway. Identification of such mutations in high risk individuals with family history, has led to preventive treatment options, which include prophylactic bowel resection. Given that FAP has almost 100% risk of future CRC, such preventive strategies have decreased the risk for future CRC by 55%, an example of the benefits of personalised medicine.

Additionally, personalised medicine has shaped the management of breast cancer. The Herceptin-2 (HER-2) gene regulates cell proliferation and is overexpressed in 20-25% of breast tumours [99]. HER-2 positive tumours have a poorer prognosis compared to HER-2 negative, however they respond better to Transtuzumab, a humanised IgG1 monoclonal antibody chemotherapy.

In the case of CRC, as previously discussed, KRAS is a proto-oncogene involved in cellular response to extracellular stimuli. KRAS mutations can downregulate the MAPK or PI3K/AKT pathways, which are protein chains that connect the EGFR receptors with nucleic transcription. This means that KRAS mutant tumours will not respond to anti-GFR chemotherapy. Cetuximab is a monoclonal antibody which acts against EGFR inhibitor and has been widely used as chemotherapy agent in CRC; hence KRAS mutant tumours will not respond in such treatment [78]. In chapter 4 we discuss in detail this pathway and its importance in CRC.

The vision of personalised medicine is to create therapeutic protocols tailored and adapted to certain distinct molecular characteristics of each tumour. This would optimise prognosis stratification, as well as treatment plan; in simple words to avoid undertreating an aggressive tumour, but at the same time to eliminate drug-related toxicity in the case of unnecessary administration (overtreatment). There have been many efforts which attempted to develop a molecular classification model of CRC, which would essentially take over the traditional TNM classification, and achieve a more individualised approach for each case. As we discuss later on in detail (Chapter 4), Simons et. al (2014) [82] suggested a novel classification model based on MSI, CIN, and CIMP; this incorporates further point mutations including KRAS or BRAF

genes, and stratifies prognosis based on those features. Similarly, Domingo et al. (2014) [80] developed a mutual concept which goes into a more detailed description of 7 distinct molecular subtypes of CRC. In both cases there is common ground, however there is still a long way to reach consensus and subsequently clinical applicability. Despite being discussed for more than a decade, personalised medicine, especially in the case of CRC prognosis and treatment has yet to reach the clinical applicability and the consensus that most enthusiastic researchers would expect.

Nevertheless, personalised medicine follows a rapid evolution, and newer evidence, along with advanced technology based on artificial intelligence becomes available [102]. Therefore, there is hope that increasing of the understanding CRC biology with implementation of novel technology (artificial intelligence) will soon revolutionise the existing knowledge to a powerful, and clinically applicable, molecular classification model of CRC; this would allow individualised approach for each case.

3 Our Research Question: Can Oncogenes Predict Recurrence After TEMS?

Our research question is whether the aforementioned panel of molecular biomarkers can predict local or distant recurrence of early rectal cancer following Transanal Endoscopic Microsurgery; we also explored the association between those biomarkers and cancer-related death. Finally, we aimed to define the association of those biomarkers with several clinical and pathological features that are generally known to play a role in the prognosis of rectal cancer.

4 Material and Methods

As this dissertation is based on a "thesis by publication" template, the materials and methods of each study are extensively described in dedicated chapters, which have been previously published. In this chapter, we provide a synopsis of the basic characteristics of the cohorts that were used to produce our results. Also, we explain the rationale behind each study, and finally proceed to briefly describe the standard operational procedures followed by the Advanced Diagnostics Laboratory at KCH.

4.1 Molecular Biomarkers and Classification Model in the evaluation of the prognosis Colorectal Cancer [78]

Initially, we performed an extensive narrative review of the literature. The aim was to collect all the available evidence across the literature and define those biomarkers that could be applicable to our research question. As part of the same review, we divided all available biomarkers into those that were prognostic and those that were predictive, the definition of which has been discussed in the Introduction section. Further, we additionally delineated and summarised the accepted molecular mechanism by which each of the biomarkers was associated with prognosis or response to therapy. Although by definition this was a narrative review, a systematic approach to evaluate the literature was followed to ensure all available studies were analysed. The keywords used included "colorectal cancer," "molecular biomarkers," "MSI," "CIMP," "CIN," "KRAS," "BRAF," "miRNA," and "review." The keyword strategy was based on previous narrative reviews and aimed to include any biomarker previously discussed in the literature as an essential element of CRC molecular biology.

Careful selection and appraisal of the studies were performed to select those that were suitable and applicable to our review, with sample size taken into account to maintain quality of analysis. We also manually searched each study, methodically appraising the relevant studies that were cited and discussed. Our analysis focused on a 5-year period from 2009−2013, with extensive involvement of papers derived from a basic science background that had been the basis for large multinational studies.

This manuscript was published in the journal "Anticancer Research" [34: 2061-2068(2014)].

4.2 BRAF V600E mutation in colorectal cancer is associated with right-sided tumours and iron deficiency anaemia [103].

This is a pilot study based on some of the biomarker panels discussed in our review. The cohort of the population consisted of suspected CRC cases referred by general practitioners to KCH via the 2-week-wait pathway. Suspected CRC cases are primarily screened by GPs using a form specially designed to include all the possible CRC symptoms, as well as demographics and family history (FH) of CRC. Although this form is not standardised across the UK, it appears to include similar criteria across the country.

We retrospectively identified 1,446 regional referrals of suspected CRC within a 4-year period (2011-2014). We identified 41 confirmed CRC cases, of which 21 were male and 20 were female (mean age 67.99, SD= 13.5).

Data were retrospectively collected in a predesigned Microsoft Excel spreadsheet and classified for demographics; symptoms at referral (based on the standardised referral form); outcome of investigations performed including imaging and scoping; histopathological features; as well as biochemistry results, including Hgb levels. The presentation profile (symptoms at presentation) included "changes in bowel habits−CIBH;" "weight loss;" "rectal

bleeding;" "unexplained iron deficiency anaemia-IDA;" "abdominal mass on examination by GP;" and "family history of CRC." All data were collected from the electronic patient record (EPR), which included all the above-mentioned information, as well as scanned GP referral forms and clinic letters. Molecular biomarker data were available, including KRAS and BRAF status, which were collected from the EPR and from WinPath (the KCH pathology database), which imports data from the Advanced Diagnostics Lab at KCH. All the cases were managed in an MDT environment and discussed in the relevant MDT meeting prior to any decision being reached. Data were analysed with IBM SPSS for Macintosh, version 22.0 (IBM Corp., Armonk, NY, USA) using the t-test and ANOVA test. Finally, cross-referencing of our results was performed using existing data in the literature.

The aims of this pilot study were to explore the association of KRAS and BRAF status with the presentation profile, and to explore the association of KRAS/BRAF with histopathological features and biochemistry profile at presentation. Finally, we explored the difference in the expression of KRAS and BRAF across different stages of CRC and location of cancer.

This study was published in the journal "Anticancer Research" [35: 2345-2350 (2015)], and was an invited submission following presentation of the concept at the relevant international conference. Results are presented in the relevant chapter.

4.3 KRAS mutant status, p16 and β-catenin expression may predict local recurrence in patients who underwent transanal endoscopic microsurgery for stage I rectal cancer [104].

This is the main cohort based upon which we studied our biomarkers. Primarily, this is a case-control study in which we explored the difference between recurrent and non-recurrent early

rectal cancer (Stage I) following TEMS. Our institution performs TEMS for early-stage rectal cancer according to national UK guidelines. All decisions are taken in the MDT meeting which takes place on a weekly basis, and there are dedicated slots for early rectal cancer cases. All the cases were operated on by the same surgeon (SP), who is the first supervisor of this dissertation. Preoperative staging of the specimens was based on ERUS and magnetic resonance tomography (MRI) with a special pelvic protocol. The diagnostic accuracy and rationale behind this is discussed extensively in the Introduction. A particular follow-up protocol was in place for 5-year surveillance for early diagnosis of recurrence. This included 6-monthly endoscopy, CEA levels, MRI pelvis, and CT abdomen/pelvis. This schedule was reduced to annually after the fourth year.

From a 10-year cohort at KCH, we selected Stage I cases with the aim of achieving an average 3-year follow-up window. Of the selected cases, 19 were non-recurrent and 10 were recurrent. This was to ensure that adequate samples of recurrent and non-recurrent lesions were available for comparison purposes. Given the limitations of the number of the entire KCH TEMS cohort at that time (48 cases in total), this was a relatively good sample size.

Radiological staging took place before and after neoadjuvant radiotherapy where applicable. Decision for neoadjuvant or adjuvant radiotherapy was taken on the basis of patients' fitness for surgery as well as on the basis of the patients' choice. The option for completion surgery was offered in individualised cases, but this was declined by the patients and hence it was not performed. Demographics and follow-up outcomes of MRI, endoscopy, and blood results were prospectively collected. Histopathological features from endoscopic biopsies and theatre samples were also prospectively collected.

The molecular biomarker panel, including KRAS, BRAF, p16, β-catenin, MGMT, and mismatch repair (MMR)/MSI were retrospectively analysed by the research student (MS), who was directly supervised by staff from the KCH Advanced Diagnostics Laboratory. The

majority of the samples were directly analysed by Dr Jane Moorhead (JM), the laboratory director, with MS being present for familiarisation with the relevant standard operational protocols (SOPs). Original photos from the microscope were obtained of various aspects of the immunohistochemistry (IHC) in order to supplement this manuscript. The methodology for the molecular techniques (pyrosequencing, PCR, and IHC) is discussed in detail later on in this chapter.

As a general cohort description, 18 patients were male and 11 were females. Mean age at diagnosis was 67.93 years (28.25 - 86.87). All the specimens were deliberately chosen as Stage I, which represents the vast majority of the entire TEMS cohort at KCH.

Statistical analysis of the results was performed by MS under supervision, using IBM SPSS for Macintosh, version 22 (IBM Corp., Armonk, NY, USA). We performed bivariate correlations (Pearson's and Spearman's rho, as well as chi-square associations) to identify any potential links between the follow-up parameters and KRAS, BRAF, p16, β-catenin or MGMT. Logistic regression analysis and Kaplan-Meier curves were not possible due to limitations in the sample size.

Our primary aim was to compare the association of the panel of biomarkers, including KRAS, BRAF, p16, β-catenin, and MMR/MSI, with the survival outcomes including recurrence and cancer-related deaths. As part of this study, we explored the association of the status of biomarkers against the time-to-recurrence. Also, we aimed to compare the status of our biomarker panel against histopathological features and demographics.

4.4 KRAS mutant status may be associated with distant recurrence in early-stage rectal cancer [77].

A recommendation from the mid-term Viva review was to include a rectal cancer series from KCH in order to compare the significance of the already studied panel of biomarkers. One

hundred and thirty-five consecutive rectal cancer cases, from 2011−2014, along with prospective collection of clinical and pathological data were included in the study cohort. Prospective collection of data was part of a UK National Bowel Cancer Audit.

All the cases were discussed in the cancer MDT, which is held on a weekly basis at KCH, for treatment stratification. Intense follow-up on a 6-monthly basis according to the local guidelines included 6-monthly endoscopy, CEA, CT abdomen pelvis and thorax, and occasionally MRI pelvis and ERUS.

Data were collected on demographics, preoperative staging including imaging, MDT outcomes, preoperative biopsy where applicable, and theatre specimen histopathology. Biochemistry profile and treatment stratification (neoadjuvant/adjuvant chemo/radiotherapy) data were also collected. All data were stored on the EPR and accessed online from hospital-based computers.

Analysis of biomarkers was performed using the same SOP protocol, led by Dr S Diaz-Cano and Dr Jane Moorhead. A description of processing of the sample is given in the next Methods section. Selection of biomarker panels was based on the same selection criteria as those for the TEMS cohort.

The primary aim of the study was to explore the prognostic significance—against cancer-related survival and local or distant recurrence—of the same biomarkers studied in the TEMS cohort, in cases of a cohort of patients who underwent radical surgery. Also, we aimed to further compare the significance of those biomarkers in the particular subgroup population of Stage I rectal cancer specimens and to identify any associations between those biomarkers and histopathological features including LVI, PNI, stage of disease, etc.

A total of 135 cancer cases were identified in our cohort (mean age at diagnosis was 64.67 years, range=22−89 years, SD=13.32); 87 cases were males and 48 were females. The mean follow-up time was 39.21 months (range=5−83 months, SD=21.34). Twenty-one cases were

ASA grade I, 72 ASA grade II, 35 ASA grade III, and 1 was ASA grade IV. With regard to the preoperative MDT stage, 28 cases were Stage I, 30 were Stage II, 45 were Stage III, and 31 were Stage IV. The stage was defined during the MDT meeting based on either radiological or preoperative biopsy results, if available. Neoadjuvant/adjuvant therapy details are given in the relevant chapter section.

Statistical analysis of our results was performed using IBM SPSS for Macintosh version 22 (IBM Corp., Armonk, NY, USA). Univariate correlations (Pearson's and Spearman's rho as well as chi-square crosstabs) were used to identify any potential links between various parameters and KRAS, BRAF, p16, β-catenin, or MGMT. Independent t-test associations were used to compare the means of different groups. The statistical significance level was set at p=0.05. Subgroup analysis of the Stage I rectal cancer cohort was undertaken to evaluate and compare the significance of the panel of biomarkers with the TEMS cohort.

This study was published in the journal "Anticancer Research" [37 1349-1358 (2017)].

4.5 Biomarker analysis - Synopsis

All biomarkers were assessed on formalin-fixed, paraffin-embedded (FFPE) samples. Beta-catenin, MMRs, MGMT, and p16 assays were performed using IHC. Four-μM sections of the tumour were cut onto coated slides, and IHC was performed using standardised protocols for each antibody. The final step was visualisation of antigen-antibody complexes by the addition of the chromogen, diaminobenzidine. The slides were then assessed (by SDC and JM) and scored for percentage of tumour cells and protein location figures 13-19.

KRAS mutation analysis was performed on the same tumour samples. HH&E-stained sections of the tumour were assessed and marked for tumour content, and the tumour was then macrodissected using serial, unstained sections from the non-tumour components, allowing for

enrichment of tumour cells. DNA was then extracted from these samples using standardised protocols and quantified by Qubit analysis. Polymerase chain reaction (PCR) was performed using primers on either side of the regions of interest: codons 12 (35G>A, 35G>T, 34G>T), 13 (38G>A), and codon 61 (182A>T). After immobilisation of the resulting amplicons, single-stranded DNA was prepared and the sequencing primer for each region was annealed. The samples were then analysed on a Qiagen Q24 pyrosequencer©, using the appropriate software (PyroMark Q24 software, version 2.07). The mutation status of each tumour was reported according to standard protocols (by SDC and JM). Appropriate positive and negative controls were included for all assays.

Photos were obtained by JM with the relevant permission of the Advanced Diagnostics Laboratory (King's College Hospital NH Foundation Trust, London, Denmark Hill, UK).

4.5.1 Immunohistochemistry (IHC) on FFPE samples (p16, MGMT, β-catenin, MMRs)

Immunohistochemistry is a technique used to identify [105] and analyse protein expression in the context of tissue morphology. The basic concept of IHC is based on the principle of antibodies that bind into their target epitope and at the same time are identifiable via a chromogenic or fluorescent readout. The steps followed for IHC in the Advanced Diagnostics Laboratory at KCH are based on those provided by the commercial supplier and can be summarised as follows [106]:

- preparation of solutions and reagents
- sample preparation
- deparaffinazation/rehydration
- antigen unmasking
- staining

- dehydrating and mounting sections

Preparation of solutions and agents: This is to ensure that the following are available: ethanol, anhydrous denatured; xylene; hematoxylin; wash buffer; antibody diluent options (SignalStain®, TBST/5%, PBST/5%); antigen unmasking options (EDTA, citrate, TE, pepsin); 3% hydrogen peroxide; blocking solution (TBST/5% normal goat serum); detection system (SignalStain®); and substrate (SignalStain® DAB Substrate Kit 8059).

Sample preparation: Paraffin-infiltrated tissue is briefly cooled in a mold with liquid paraffin in order to immobilise the tissue. The base of a cassette is placed on top of the mold, then filled with liquid paraffin and cooled. Four-mm slices from the microtome are baptised in a water bath. Mount sections are left to dry overnight onto the charged slides.

Deparaffinization/rehydration (figure 6): Prior to antibody masking, paraffin has to be removed by putting the sections on 3 containers of xylene, 5 minutes each. The sample needs to be rehydrated using 2 containers of 95% ethanol, and finally the sections are washed twice in dH2O for 5 minutes each.

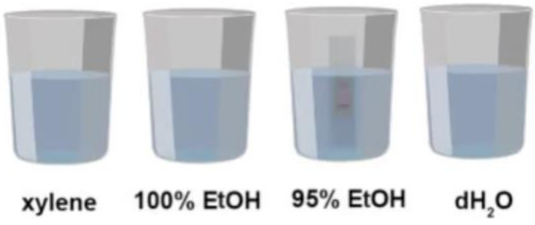

Figure 6- Deparaffinization/ Rehydration

Antigen unmasking: Prior to antibody staining, we have to break the cross-links formed by formalin fixation using either citrate, EDTA, TE, or pepsin.

Staining: We performed chromogenic staining using SignalStain® diaminobenzidine (DAB) solution. The sections were washed in dH20 3 times for 5 minutes. Sections were placed in 3% hydrogen peroxide for 10 minutes. Then, sections are washed initially in dH2O for 5 minutes each and in wash buffer for 5 minutes. 100−400 µL of blocking solution were used to prevent nonspecific blocking. The blocking solution is then removed and 400 µl of the primary antibody solution is used for each section. The sections are rested overnight at 4°C in a humidified chamber. The next step includes equilibration with SignalStain® boost detection reagent. The antibody solutions are removed and the sections are washed in wash buffers 3 times. Three drops of SignalStain® Boost detection reagent are used to cover the section and then rested for 30 minutes in a humidified chamber; 100−400 µl of SignalStain® DAB for 10 minutes on each section is enough for acceptable staining intensity. The slides are then washed with dH20.

Dehydration and mounting of sections: Either ethanol or xylene is used for this purpose.

The slides are assessed by Dr Jane Moorhead or Dr Salvador Diaz-Cano with MS to score for percentage of tumour cells and protein location. The original (electron microscope) pictures are available below.

4.5.2 Polymerase chain reaction (PCR) – KRAS and BRAF mutation status

KRAS and BRAF mutation analysis was performed on the same tumour samples. The HH&E−stained sections of the tumour were assessed and marked for tumour content, and the tumour was then macrodissected using serial unstained sections from the non-tumour components, allowing for enrichment of tumour cells. DNA was then extracted from these samples using standardised protocols and quantified by Qubit analysis.

Reagents and equipment: [107]

- DNA extraction buffer

- isopropanol
- 70% (v/v) ethanol
- table-top microcentrifuge

The tumour microdissected material with unstained sections of the non-tumour components were placed into sterile Eppendorf tubes and mixed with 400 µl of DNA extraction buffer using the vortex (13.000 rpm for 1 min). The supernatant was transferred to a new Eppendorf tube and mixed with isopropanol using the vortex (13.000 rpm for 1 min). The supernatant was discarded, and the DNA pellet was washed with 70% (v/v) ethanol using the vortex (13.000 rpm for 1 min). The pellet (DNA) was inverted onto a paper towel and then air-dried to remove remaining ethanol. Finally, the DNA was dissolved in 100 µl ddH2O and stored at 4°C for immediate use.

DNA amplification and sequencing reaction [107]:

Thermal cycling took place in 0.2 ml thin-walled PCR tubes with 50 µl reaction volume. Contents of the reaction solution were: 1µl of 10 pmol universal barcode primers [108]; 1µl template DNA; 5µl 10x PCR buffer; and 0.4 µl Thermus aquaticus polymerase. We used the following conditions as described by Margam et al. [107]: 95°C/2min, then 5 cycles (95°C/30 sec each cycle), 45°C for 45 sec, 72°C for 1.5 min, and then followed by 31 cycles (95°C, 30 sec), 50°C for 45 sec, 72°C for 45 sec, and a final cycle of 72°C (8 min).

4.5.3 Pyrosequencing Qiagen Q24 Advanced Technology (Qiagen Q24® - qiagen.com)

Pyrosequencing was performed using Qiagen Q24® *[109]*, following the local standard operational procedures to identify KRAS codon 12 (35G>A, 35G>T, 34G>T), 13 (38G>A), and codon 61 (182A>T) [104] mutations, as well as BRAF (c.f. 1799 T>A) [76, 95, 103] V600E mutation.

The essential pyrosequencing workstation is presented in figure 7. Reagents; vacuum preparation workstation; instrument (Pyromark Q24 Instrument) [110] (figure 8); and application software (PyroMark Q24 software) are essential for further processing of identification of KRAS and BRAF mutations. PyroMark Q24 Technology [109] (figure 8) allows 1−24 gene specimens to be analysed in 15 minutes.

The first step is sample preparation, which is taken over by PCR amplification. PCR products are cleaned to remove any remaining primers or nucleotides, and DNA is quantified by Qubit analysis. The vacuum preparation workstation is essential for cleaning the PCR DNA in order to end up with a single-stranded DNA.

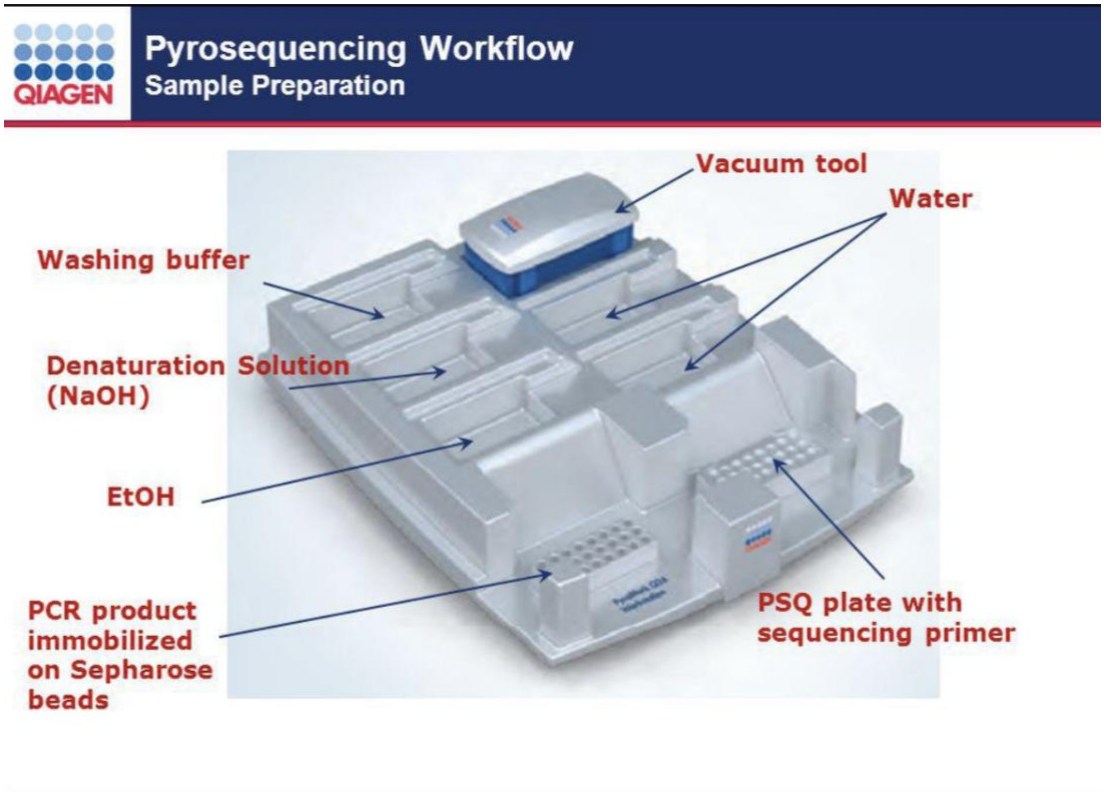

Figure 7 - Pyrosequencing Preparation Workstation

A bottle with Sepharose beads is essential for immobilizing the PCR product to beads. A 2 µl/sample of Sepharose beads solution, 40 µl/sample of binding buffer solution, and Milli-Q 18.2 MΩ x cm or equivalent (18−28µl/sample) are mixed with 10−20 µl of PCR product in a

cubicle (80 µl in total). The solution is agitated constantly for 5−10 minutes at 1.400 rpm. The final solution is processed according to the steps on the vacuum preparation workstation (figure 3) in order to separate DNA strands into a single-strand product ready for pyrosequencing.

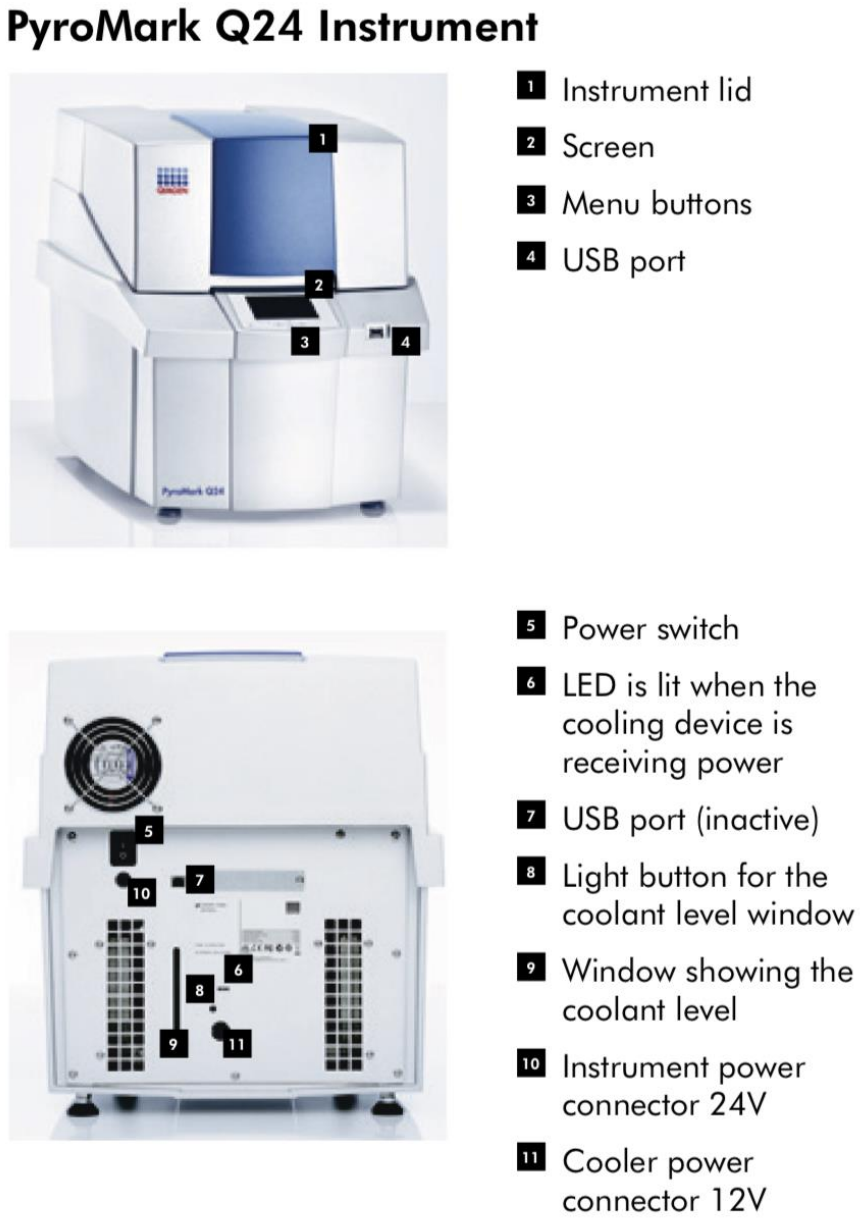

Figure 8 - PyroMark Q24 Instrument

The pyrosequencing principle uses sequencing by synthesis for accurate and quantitative analysis of DNA. The principal algorithm of pyrosequencing consists of 9 steps. The

description below is based on the manufacturer's principal manual of the device which we used for our specimens [111]. Initially, a sequencing primer is hybridised to a single-stranded, PCR-amplified product template (process described previously). Then, the template is incubated with enzymes and substrates, and the first of the 4 nucleotides are added into the reaction. The nucleotides are incorporated into the complementary bases of the template strand. Each incorporation is a chemical reaction which releases pyrophosphate (PPi) based on the following reaction: $(DNA)_n + dNTP \rightarrow (DNA)_{n+1} + PPI$ (facilitated by DNA polymerase). The released PPI is converted to ATP by the ATP sulfurylase in the presence of adenosine 5' phosphosulphate. ATP is essential for converting the luciferin into oxyluciferin by the enzyme luciferase, which is the principle behind pyrosequencing. Conversion of luciferin to oxyluciferin produces light that is picked by charge-coupled devices (CCD), which is seen as a peak in the pyrogram (figure 9).

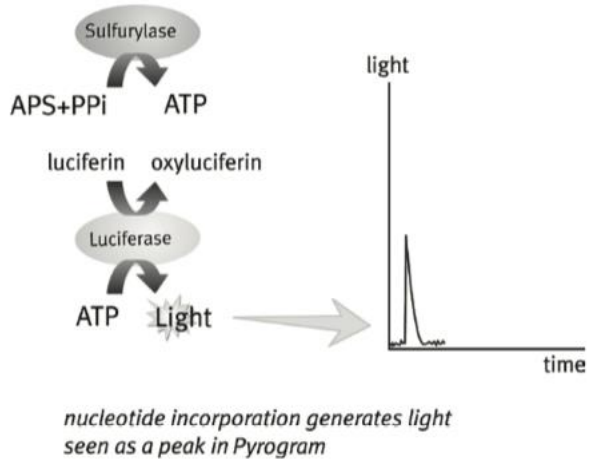

Figure 9: Conversion of luciferin to oxyluciferin produces light which is picked by CCD

Apyrase is responsible for continuously degrading unincorporated nucleotides (dNTP) → dNDP + dNMP + phosphate. Apyrase also degrades ATP to ADP + AMP + phosphate. Nucleotides are added one at a time, and as the process continues, the nucleotide sequence of the complementary strand is determined by the peak in the pyrogram (figure 10).

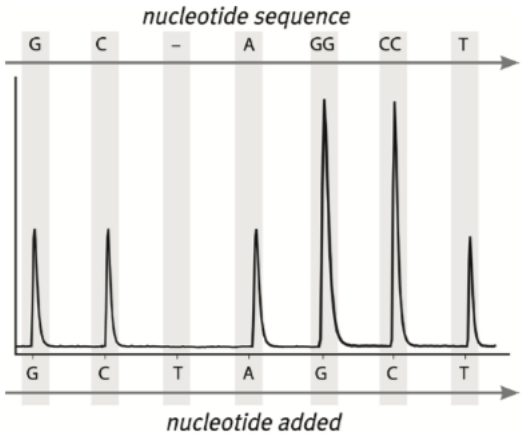

Figure 10: Nucleotide Sequence (dNTP sequence)

PyroMark Q24® [109] software is used for visualisation of the nucleotide sequences, which are identified and confirmed by the experts (JM & SDC).

5 Molecular Biomarkers and Classification Models in the Evaluation of the Prognosis of Colorectal Cancer – [PMID: 24778007]

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5.1 Abstract

Colorectal cancer (CRC) is the second leading cause of cancer-related death. Despite the progress that has been made towards the identification of the molecular mechanisms involved in CRC, there are many unclear points. The current opinion is that microsatellite instability (MSI), CpG island methylator phenotype (CIMP) and chromosomal instability (CIN) seem to play a significant role. MSI is related to point mutations in defect mismatch repair system of DNA. There are two well-established MSI phenotypes: MSI-high (MSI-H) and MSI-low (MSI-L or MSS). CIN refers to a different cellular event which originates from the presence of an abnormal chromosome complement or number. CIMP is the third most commonly involved event, and is defined by widespread methylation of CpG islands of suppressor promoters, with two phenotypes: CIMP-high and CIMP-low which interact with MSI or CIN status

V-raf murine sarcoma viral oncogene homolog B (BRAF) is a serine-threonine protein kinase that acts as a downstream effector of the Kirsten rat sarcoma viral oncogene homolog (KRAS) pathway. Various studies have revealed that BRAF V600E mutations appear to be a valid indicator of poor prognosis. KRAS is a proto- oncogene which encodes a GTP-ase involved in cellular response to extracellular stimuli. Its prognostic value is still controversial. However, wild-type KRAS is associated with better response to Endothelial Growth Factor Receptor inhibitors combined with standard chemotherapy. Loss of Heterozygosity, especially involving 18q, is a well-known potential mechanism for tumorigenesis that has been studied in CRC. Vascular Endothelial Growth Factor is a proangiogenic factor linked with the aggressiveness of CRC. Emerging data show that Cycloxygenase 2 overexpression is significantly associated with worse outcomes in CRC. Recent studies highlight microRNAs as promising prognostic biomarkers. More specifically, the down-regulation of miR-451, miR-625, miR-29c, miR-126, miR-129 and miR133 is purported to be a poor prognostic factor, while miR-224 was overexpressed in CRC.

Key Words: CRC biomarkers, CIMP, CIN, MSI, KRAS, BRAF, miRNA, review.

5.2 Introduction

Currently, treatment strategies and clinical outcomes in colorectal cancer (CRC) are determined by cancer stage as defined by TNM or relevant staging protocols [112], based on local tumor's penetration and spread to lymph nodes or other organs. The cornerstone of non-metastatic CRC treatment remains the surgical resection. In patients with higher stage disease, (III or high risk II) there is a significant risk of reoccurrence. Although certain histological factors such as differentiation, Grade, lymphovascular invasions have been identified as exposing to higher risk there is still lack of understanding of molecular factors which may affect the risk of metastasis and recurrence.

In recent years, molecular biomarkers have generated interest as prognostic markers or in the case or as in the case of KRAS, as factors involved in the treatment choice. Generally, a biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological response to a specified therapeutic intervention. Biomarkers can be either prognostic or predictive [69, 70, 113, 114] . Prognostic values are defined by the estimated life expectancy post diagnosis and treatment whereas predictive biomarkers are related to the response of a patient to a relevant treatment strategy.

The purpose of a molecular classification is to identify similar characteristics among individual tumors, and then predict empirically the pathogenesis and biological behavior of a particular tumor [71, 72, 115-117] [71]. The most accepted way of creating a classification model is to identify and correlate single cellular events that have been statistically proven to play a role in the tumor genesis.

The starting point of these efforts was the identification of significant stages in the progression from healthy epithelium to cancer [83]. Currently some theories exist which explain the transition from adenoma, the first pre-cancerous type of lesion, to carcinoma and then invasion to other the tissues. In this way, commonly involved events in CRC were identified and highlighted as the guiding points for a classification model. Subsequently, the first efforts to summarize and correlate these events were published, but none was actually adequately evidence-based to achieve translation to clinical practice

Most of the current molecular classification models are based on MSI, CIN and CIMP and they correlate these event with other significant mutations *i.e. KRAS*, and *BRAF* [84, 118].

In this review article, we summarize what is currently believed in terms of all the popular potential biomarkers in CRC and we are going to set the parameters up for further discussion and points of interest for further research

5.3 MSI

Microsatellite disorders are established as frequent event in CRC, occurring in around 22% of cases [56, 119]. There are many reports supporting the effect of MSI CRC prognosis [70, 80, 114, 120] [121-124] [72, 116, 117, 125, 126]

Microsatellites are short repetitive DNA nucleotide sequences which are prone to frame-shift mutations and base-pair substitutions during DNA replication [70, 114]. They are involved in the DNA repair system and their mutations were initially associated with Lynch Syndrome (LS), which is an autosomal dominant disorder, highly associated with 3% of multiple CRC types. MMR genes involved in Lynch Syndrome are MLH-1, MSH2, NSH6 and PMS2 [81] . Loss of these genes results in defective MMR, which in turn results in MSI.

Sporadic CRC is more likely to be associated with hMLH-1 mutation. In terms of MSI status classification most of the studies divide it into three categories: MSI-high (MSI-H) at ≥30%, MSI-low (MSI-L) at 10-30% and microsatellite stable (MSS)[70, 81, 114] . Some other studies

aim to simplify MSI status as being positive or negative[80, 82, 127]. It been questioned whether MSI-L exists or falls in the same category as MSS [128].

Most of the studies aiming to establish a classification model in CRC, consider MSI status as one of the primary criteria for categorization [80, 127] (Table 6). A recent multivariate analysis identified MSI alterations in 119/892 CRC samples and highlights MSI status as one of the major prognostic biomarkers [80] . Another interesting study (Simons et al. 2013) suggested a model which is mainly based on MSI status and its relationship with CIMP and CIN. In the same study, it was noted that APC and KRAS mutations were less frequent in MSI tumors (13.3% and 10.9% respectively) whereas BRAF V600E mutation is most often seen in MSI tumors (58.7%) [82]

There are many clinicopathological variables associated with MSI status. For instance, patents with MSI-H tumors have in general greater 5-year overall survival irrespective of stage compared to those with MSI-L and MSS tumors. The PETACC III trial has confirmed these retrospective findings and suggested that MSI-H is a strong prognostic factor for relapse-free and overall survival in patients with stage II and III [70, 114]. Moreover, MSI-H tumors have been classified as proximally located, poorly differentiated with a higher incidence in female gender. They are also characterized by mucinous differentiation, increased age at onset and round and vesicular nuclei with a prominent nucleolus[69, 80-82, 113, 127]

MSI	**Summary of basic features**
	appears in around 22% of CRC cases [80, 119]

MSI-positive (high)	Greater 5-year survival, proximal location, poor differentiation, female gender, mucinous differentiation [69, 70, 80-82, 127]
MSI in sporadic Colorectal Ca	Association with hMLH-1 [70, 81]
MSI in LS	Association with MLH-1, MSH2, MSH6, PMS2 [81]
Correlation with KRAS/BRAF mutations	BRAF V600E is associated in 58.7% with MSI +ve CRC [82]
	KRAS mutations appear in 10.9% of MSI +ve CRC [80]
Predictive value	Predictive value is still controversial [69, 127]

Table 6: Baseline characteristics of MSI tumors. This table provides a summary of the baseline characteristics of MSI tumors and their correlation with KRAS and BRAF mutations

However, contrary to the promising results in terms of the prognostic value of MSI status, no clear relationship between MSI status and response to neoadjuvant chemotherapy has yet been proven. However, Ribic et al.[119] study concluded that MSI-H status can be associated with poor response to 5 FU based chemotherapy compared to MSI-L and MSS tumors[69, 113]. Nevertheless there are multiple studies with conflicting results in terms of the predictive value of MSI status and thus this is still controversial and under discussion [127] .

5.4 CIMP

Most studies develop the CRC classification model based on CIMP status and its correlation to CIN and MSI[72, 81, 82, 115-117, 127, 129] . CIMP is defined as hypermethylation of CpG island promoter. CIMP results in transcriptional silencing of specific tumor-suppressor and DNA repair genes, including MLH-1[82] .Some studies describe CIMP status as CIMP-high, CIMP-low and CIMP negative [81] . There is a discussion about whether CIMP low and CIMP negative fall into the same category[129], resulting in two categories, CIMP positive and CIMP negative; we use this for simplicity here.

There have been some reasonable studies which associate MSI and CIMP status along with Chromosomal Instability (CIN), resulting in a classification model which attempts to identify and link macroscopic variables to molecular features [82] . A recent study, Simons et al. 2013, suggests a classification based on MSI, CIMP and CIN. According to that, MSI and CIMP-positive tumors were more proximally located (85.7% and 51.9 % respectively), whereas CIMP-positive and CIN or CIN-only tumors were distally located (distal colon to rectum 52.3% and 82.1% respectively). Triple negative tumors were located both in distal and proximal colon and in 89% of them BRAF V600E mutation was present.

Regarding CIMP-only tumors (Simons et al. 2013) the average age at diagnosis was 67.6 years, 51.9% was located proximally and the differentiation grade was II at the stage of data analysis. BRAF V600E mutation was present only in 18.5% and p53 over expression was noted in 66.7 % [82].

5.5 CIN

Tumors with CIN have chromosomal gains and losses, with or without structural rearrangements possibly reflecting an increased mutation rate and this is called aneuploidy and/or polyploidy respectively [80, 130, 131] and occurs in around 60% of CRC cases [69, 113] The mechanism underlying CIN is not yet understood. Most studies tend by definition to

differentiate MSI positive tumors from CIN, as CIN reflects to a separate mechanism of carcinogenesis [82, 127]. MSI positive and CIN positive tumours have been described, but they tend macroscopically to appear more likely MSI positive[80] . The overlap between CIN and MSI is not clear [82] . CIN positive tumors are generally associated with poor prognosis and they tend to be well or moderately differentiated [80] Simons et al, hold that CIN only tumors are more frquent in men (54.7%), 76.5% at the stage of diagnosis are grade 2 and there is subsequent p53 over expression [82]. However, BRAF V600E mutation tends not to be involved in such a mechanism of tumorigenesis [80, 82], but several studies do link KRAS mutations with this molecular phenotype [127] . CIN tumors are located more often distally. CIMP-negative and CIN positive tumors have been described [82]

Domingo et al., have described 3% of MSI-H and CIN positive tumors tending to have characteristics closer to MSI-H CIN negative tumors i.e. right-sided location and increased BRAF mutations, while p53 mutation and 17p LOH was not increased significantly, and thus there was no association [80].

5.6 KRAS mutations

KRAS is a proto-oncogene which is involved in cellular response to extracellural stimuli [70, 114, 132]. In cases of KRAS mutations there is a structural activation of downstream signaling pathways i.e. MAPK and PI3KAKT pathways[69, 113] , and through this mechanism tumour cells are more resistant to inhibition of surface receptors of tyrosine kinase such as EGFR. The MAPK pathway was recently found to be one of the fundamental mechanisms that get involved in the sequence of tumorigenesis [56, 119]

Point mutations in codons 12, 13 and 61 of KRAS gene are noted in about 30-40% of CRC[69, 113] and in total there have been identified around 85 KRAS mutations have been described[56, 119]. These different molecular phenotypes of KRAS may result in different pathways of carcinogenesis which could alter the macroscopic phenotype.

However, it is still controversial whether KRAS can be considered as a valid biomarker or not, as recent studies suggest there are no clinicopathological feature to support this [56, 70, 114, 119]. There are some controversial data emerging that link KRAS mutation with poor prognosis. Phipps et al. found KRAS mutation to be associated with poorer prognosis compared to wild-type KRAS [90] . Most studies link KRAS mutation with certain molecular phenotypes i.e. CIN whereas they are less likely to be noted in MSI positive tumors[70, 80, 82, 114, 127]

An interesting study in Moroccan patients with advanced CRC showed that [133] 76.09% of patients had wild type KRAS genotype, whereas 23.91% were KRAS mutants, The majority of KRAS mutations referred to an amino acid substitution of glycine by aspartic acid (68.2%)

On the other hand, despite the debating prognostic value of KRAS, it has been established that wild-type KRAS is associated with better response to EGFR inhibitors in terms of adjuvant chemotherapy setting i.e. cetuximab [56, 70, 76, 112, 114, 119, 134-136]. An interesting study by DiBartolomeo et al. in 2013 concluded that patients with KRAS/NRAS, BRAF and TP53 wild-type tumours could had the maximum benefit from treatment with cetuximab, oxaliplatin and UFT[137] Nevertheless, Selcukbirik et al. 2013 found that BRAF and KRAS mutations do not seem to be a potential biomarkers in the treatment of metastatic CRC (mCRC) with bevacizumab therapy [138]. Also, Bruera et al. also noted a worse prognosis in patients with metastatic CRC and KRAS (c35 G) / BRAF mutations [139], and a similar hypothesis is supported by Loupakis et al. 2013 where EGFR ligand was significantly modulated by cetuximab plus irinotecan therapy[140]

Tian et al 2013 states that a combined signature of KRAS/BRAF and PI3KCA could offer an optimized source of information for cetuximab responsiveness of the tumour[141]. Saridaki et al 2011, concluded that KRAS-BRAF mutations and EREG expression can be used as biomarkers to further select patients undergoing anti-EGFR therapy[142]

5.7 BRAF mutation

BRAF gene encodes a serine-threonine kinase that acts as an inhibitor of the RAS/MAPK intracellular signaling pathway. Mutations of BRAF occur at early stage of colorectal carcinogenesis[94] .

In terms of correlations of BRAF with the main cellular events, most studies associate BRAF V600E activating mutation with sporadic MSI-H CRCs [69, 113] [56, 70, 80, 82, 114, 119] and it is linked with poor prognosis [143] (table 7). What is more, no association between BRAF mutations and CIN has yet been noted [69, 80, 82, 113]. According to Doming et al., BRAF alterations occur in 10% of CRC cases and there is a negative association with KRAS mutations[80], whereas there is primary positive association with MSI and CIMP-high [115, 117, 136] .

In a recent study (Lochhead et al. 2013) *BRAF* mutation, occured in 10% to 20% of CRC and there was associated with MSI-high through its relationship to CIMP-high [122] . The same study concludes that BRAF mutations are associated with inferior prognosis. Eklof et al. analyzing the mutation status in KRAS and BRAF and PIK3CA and PTEN expression in two separate CRC cohorts, state that KRAS and BRAF status are important in the establishment of the prognosis in CRC and should always be considered[144] . Similarly, Yokota et al. conclude that mutated BRAF is one of the most powerful prognostic markers in CRC[145] .

Nevertheless, BRAF as a predictive marker is still under discussion [76, 114]. There are some studies which tend to use a signature of BRAF/KRAS as a predictive factor for the response to EGFR inhibitors [137-142]. Another interesting meta-analysis, [146] , taking into consideration 21 trials including 5229 patients, conclude that BRAF mutation is a predictive biomarker for poor prognosis in patients with metastatic CRC undergoing therapy with anti-EGFR monoclonal antibody, especially in those with wild type KRAS.

Finally Mesteri et al., concluded that BRAF V600 mutation can be used as a classification criterion for the evaluation of serrated lesions and progression to sessile/serrated adenoma polyps and CRC.

Gene	**wt/mutant**	**Baseline Characheristics**
KRAS	Wild-type	Better response to EGFR inhibitors [70, 76, 112, 119, 134-136]
	mutant	Most likely at codons 12,13,61 [69] In total 85 kRAS mutation have been identified and occupy around 30-40%of CRC
BRAF	V600E mutation	Predictive value is still under discussion [70, 76] BRAF mutations are related with 10-20% of CRC (Lockhead et al.) and they are associated with inferior prognosis [122] V600E mutation is associated with sporadic MSI + tumours [69, 70, 80, 82, 119]

Table 7: KRAS and BRAF gene mutations baseline characteristics. This table describes the baseline characteristics of KRAS and BRAF mutations regarding predictive value and common mutation patterns

5.8 VEGF

VEGF is a proangiogenic factor which is involved in endothelial cell proliferation, migration and vascular permeability [114]. Increase in its expression levels is associated with poor prognosis, low response to preoperative radiotherapy, and more likelihood for recurrence [147].

Angiogenesis plays a significant role in CRC as neovascularization markers are overexpressed in most of the cancer cells [148]. VEGFA is involved in early tumour stages i.e. adenoma

formation. According to the same study, VEGFA was associated with advanced cancer stage and metastatic disease via a angio-lymphatic invasion pathway.

Moreover, there has been noted a relationship between VEGF-C and collagen triple helix containing 1 has been noted in terms of prognostic value in rectal cancer [149]. VEGFC activates the tyrosine-kinase-linked receptor of the VEGFR-3 pathway and induces the lymphagiogenesis. [148] This means that the overexpression of VEGF-C could be a valid molecular biomarker for lymphagiogenic metastasis.

5.9 COX -2 cycloxygenase 2 enzyme related pathway

Several prospective studies have shown that COX-2 overexpression could be interpreted as an adverse prognostic factor for CRC[150].

There are some studies which support that use of COX-2 inhibitors i.e. aspirin can be associated with lower risk of CRC, especially with patients who have been diagnosed with CRC in which COX2 is overexpressed[117]

There is another study [122, 123] evaluates the correlation between aspirin intake and colorectal cancer according to BRAF mutation status using biennial questionnaire on aspirin and collected data from 1986 to 2006. The study concluded that regular aspirin use was associated with lower risk of wild-type BRAF CRC but not of BRAF-mutated cancer risk.

Finally, a recent study noted [151] that positivity for COX2 and VEGF was strongly correlated with decreased DFS (p=0.007), whereas combinations of positivity for RAF kinase inhibitor protein with COX2- and VEGF was strong correlated with improved DFS.

5.10 LOH 18q

LOH especially in 18q is a well-known potential mechanism for tumorigenesis that has been studied in CRC. LOH is being an important mechanism of inactivation of tumor suppressor's genes [114] . There have been several studies trying to interpret the potential prognostic effect

of LOH 18q in CRC, however there have been inversely associated with MSI. There are many tumor suppressor genes in 18q, e.g. *DCC*, *SMAD4* (DPC4), *SMAD2*, *and* CABLES1 [72, 116, 117] which might interfere with and subsequently explain the prognostic value of LOH 18q . Nevertheless, in the same study it is clear in 555 non–MSI-high tumor samples with informative 18q LOH data, LOH was present in at least one 18q marker in 362 tumors (65%;). There was an association between LOH 18q and obesity (BMI ≥ 30 kg/m^2; p =0.018), distal colon location (p =0.025), low tumor grade (p =0.0060), low-level LINE1 methylation (p=0 .040), wild-type *KRAS* (p=0.015), and JCVT (p=0.0004). The same study concluded that there is no association with LOH 18q and prognosis as yet.

5.11 MiRNA – Developing New Promising Biomarkers

miRNAs are short, 18-25 nucleotide non-coding single-stranded RNA sequences which are involved in regulation of gene expression on post-transcriptional level, through binding to their target protein-encoding mRNA [152]. (Michocova et al.) They are believed to play an important role in the pathogenesis of colorectal cancer [153]. There are numerous studies which identify the presence of various miRNAs as predictive or prognostic biomarkers. In the study of Svoboda et al., [153], miR-215, miR-99a*, miR-196b, miR-450b-5p and LET-7e were associated with expression of thymidylate synthetase and radioresistance or chemoresistance to its inhibitors.

miRNAs are also involved in the regulation of the EGFR signalling pathway as well and thus they may have essential value in predicting the response to EGFR inhibitors. Another study [154] identified that miR-451 inhibits cell growth through down-regulating the P13K/AKT pathway and thus it potentially has a repressive role. Lou et al., [83] claimed that miR-625 may have a prognostic and predictive role in CRC as decreased levels of the latter are associated with high incidence of metastasis and poor prognosis. [155] Yang et al., identified that miR-29c as having an antitumorigenic role and preoperatively decreased levels of miR-

29c were associated with CRC relapse. miRNA-126 can have anticarcinogenic properties through inhibitition of neovascularization via blocking the 3- UTR' of VEGF [156]

Karaayvaz et al. [157] highlighted that miR-129 could potentially have a tumour-suppressor role and might be a novel target for anti CRC therapies. Dong et al., [156, 158, 159] claim that, miR-133a serves as a functional tumor suppressor in CRC via enhancing apoptosis and inhibiting cell proliferation. According to the same study miRNA 133a iincreased p53 protein and induced p21 transcription and it can serve as sensitizer to doxorubicin and oxaliplatin. One potential mechanism of the anticancer properties of miRNA 133a is the fact that it can repress RFFL-3'UTR reporter activity and reduce its protein level.Finally Liao et al. [122, 123, 158] noted that miRNA 224 can induce tumour expansion and cell proliferation through via repressing PHLPP1 and PHLPP2 genes. miRNAs seem to be an interesting field of research with promising results (table 8).

5.12 Conclusion

CRC is the second most frequent cancer in Europe and despite progress in the understanding of the molecular background of CRC, effective molecular classification remains a challenge. Facing these difficulties in the establishment of a key model, researchers have started to try different, more innovative approaches, in which gene signatures dominate. In fact, the idea of gene signatures is very promising (59, 60), but there are still several issues of reproducibility and possible overlap (61, 62). Metabolomics is a new and promising field which tends to utilise simple products from cellular metabolism in order to identify differential and pathognomonic features of cancer[160-163] . The main advantage of this approach is the easy access; however, the low specificity for CRC remains a great challenge. Nevertheless, there is much to be looked at in this field, as metabolomics could potentially be a new concept in the investigation and classification of CRC.

Thus, molecular classification of CRC still remains a challenge that researchers have to overcome in the coming years. Creating an effective classification of CRC biomarkers based on their prognostic and predictive value will generate an effective decision-making tool in everyday clinical practice

		Mechanism of action
miRNA	miRNA-451	Inhibits cell growth through the downregulation of PI3K/AKT pathway [164]
	miRNA-625	Decreased levels are associated with high incidence of metastasis [165]
	miRNA-126	Anti-cancerous action via blocking the 3-UTR' of VEGR and inhibiting the neovascularization [159]
	miRNA-129	Tumour suppressor role, potential target for therapies [157]
	miRNA 133a	Anti-cancerous properties via increase of the expression of p53 and p21 gene products [156]
	miRNA 224	

		Can induce tumour expansion via repressing PHLPP1 and PHLPP2 [158]

Table 8: MicroRNAs involved in CRC mechanisms. This table describes the main microRNAS that are involved in potential CRC pathways. Each might have different mechanisms of action by which it could result in inhibition or acceleration of tumorigenesis

6 BRAF V600E Mutation in Colorectal Cancer is Associated with Right-sided Tumours and Iron Deficiency Anaemia. [PMID: 25862899]

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Type of study: Original Research – Part of this study has been presented in the 9th International Conference of Anticancer Research in Sithonia October 2014.

*Running Title***:** BRAF V600E mutation in colorectal cancer is associated with right sided tumours and Iron Deficiency Anaemia.

6.1 Abstract

Background: BRAF gene encodes a serine-threonine kinase that inhibits the RAS/MAPK intracellular pathway. BRAF mutations occur at an early stage of colorectal cancer and their presence, 10-20% of Colorectal Cancer (CRC), is usually associated with inferior prognosis. Materials and Methods: From 41 consecutive CRC confirmed referrals from 1,446 suspected cancer cases (mean age=67.99±13.45, Male=21, Female=20), we retrospectively analyzed collected data from haemoglobin (Hb) and symptoms at presentation, location of tumor and stage of the disease, including lymphovascular invasion (LVI). Gene profile analysis data (KRAS, BRAF) were retrospectively collected and associated with the presentation profile above. Results: There was no significant correlation in presentation Hb levels and eventual disease staging ($P>0.05$ for all associations). Patients with right-sided tumours were found to have a lower Hb level than patients with either left-sided colonic or rectal tumours. Hb levels were also significantly lower in patients with the BRAF V600E mutation. KRAS status or LVI status did not have a specific correlation with Hb levels. Conclusion: BRAF V600E mutation could be associated with right-sided tumors and subsequently related unexplained iron-deficiency anaemia (IDA) at the stage of presentation. This finding may affect the choice of clinical strategy for investigation of unexplained IDA. Further research should be conducted in order to identify and support the potential biological explanation of the findings above.

Key Words: *BRAF* V600E, colorectal cancer, *KRAS*, molecular biomarkers, iron-deficiency anaemia.

6.2 Introduction

Colorectal cancer is the third most common cancer worldwide and, during the last decades, there has been a significant progress in understanding its pathogenesis [166]. Currently, there are three distinct molecular pathways to explain its complex pathogenesis [166-168]. The first one is chromosomal instability (CIN) where the aetiology is attributed to numerical (aneuploidy) or structural loss of chromosomes, whereas loss of heterozygosity (LOH) is a classic component of this theory. CIN is linked with several point mutations in genes, which are thought to be involved in the CRC pathway (*APC*, *KRAS*, *BRAF*, *etc*.) [166, 169]. The second pathway consists of a fault of the mismatch repair (MMR) system of the DNA and this is defined as microsatellite instability pathway (MSI) [8, 166, 170]. MSI is a unique feature of Lynch syndrome (LS), though it could be found in sporadic cancer as well [8]. Lastly, a newer theory is this of C-phosphate-G (CpG) island methylation (CIMP), which can result into CIMP-high and CIMP-low phenotypes. *BRAF* tends to be associated with CIMP high tumour phenotype [82, 117, 166].

V-raf murine sarcoma viral oncogene homolog B (*BRAF*) gene is involved as an inhibitor in the RAS/RAF/MAPK pathway [94, 171]. Somatic mutations in *BRAF* gene are found in 10-20% of colorectal cancer cases [122] and result in activation of *BRAF* serine-threonine kinase and subsequent up regulation of the RAS/RAF/MAPK signalling pathway [94, 172]. This can result in abnormal cellular growth, invasion and metastasis [167].

BRAF V600 E mutation is the most well studied mutation and occurs in 90% of *BRAF* mutations [172-174]. It refers to a substitution of the amino acid glutamic acid for a valine at amino acid position 600 (*c.f.* 1799T>A) [76, 95].

In terms of *BRAF* mutations and their relationship to other genetic events in CRC pathogenesis, there has been documented an association between *BRAF* mutant status and sporadic MSI-high

tumours [80, 122, 175, 176]. Lochhead *et al.* [122, 123] states that combined *BRAF* is related with MSI profile through its association with high level CpG methylator profile (CIMP) and *MLH1* promoter methylation. The same study concludes that *BRAF*/MSI status could be used as a prognostic tool [122, 123].

There are existing data on the literature, which associate *BRAF* status with certain macroscopic features. Roth *et al.* [177, 178] concludes that *BRAF* mutations are significantly associated with female sex, right-sided location, older age, high grade and MSI-high tumours. A recent metanalysis [93] concludes that *BRAF* V600E is linked with advanced TNM stage, poor differentiation, mucinous histology, MSI, CIMP, as well as female gender, older age, proximal location and mutL homolog 1 (*MLH1*) methylation.

There has been a long discussion about the prognostic and predictive value of *BRAF* mutational profile [146, 179, 180]. Recent studies associate *BRAF* mutations with poorer prognosis [122, 146] and support their role in the response to chemotherapy [76, 95, 142].

6.3 Materials and Methods

All our data derive from the urgent referral ("2 week-wait" or "2WW") Cancer Database. 2WW is a pathway where general practitioners (GP) refer suspected colorectal cancer cases to a specialized unit in the UK. We used as a source of information the original GP referral letter, as well as the Colorectal Clinic letters in order to identify the presenting symptoms of each patient. Biochemistry profile information (haemoglobin (Hb)), histology reports and molecular diagnostics were kept online on Electronic Patients' Records (EPR) database.

We retrospectively identified 41 confirmed CRC cases from 1,446 consecutive referrals for suspected cancer. From those patients, 21 were male (51.2%), 20 females (48.8%). Mean age was 67.99 years and standard deviation (SD) 13.451 (Figure 11).

Data were collected on clinical presentation profile, *i.e*. changes in bowel habit (CIBH), rectal bleeding (RB), unexplained iron-deficiency anaemia (IDA), weight loss, abdominal mass and family history (FH). Moreover, we have focused on the Hb levels at presentation, as well as tumour location and staging profile including lymphovascular invasion (LVI).

KRAS and *BRAF* mutations ware defined using the pyrosequencing technique from extracted tumour DNA. The whole series of those were performed in our Advanced Diagnostics Lab.

Data were analysed on IBM SPSS Statistics for Macintoshversion 22.0 Armonk, NY) using *t*-test and ANOVA test. Finally, cross-reference of our results was performed using existing data in the literature.

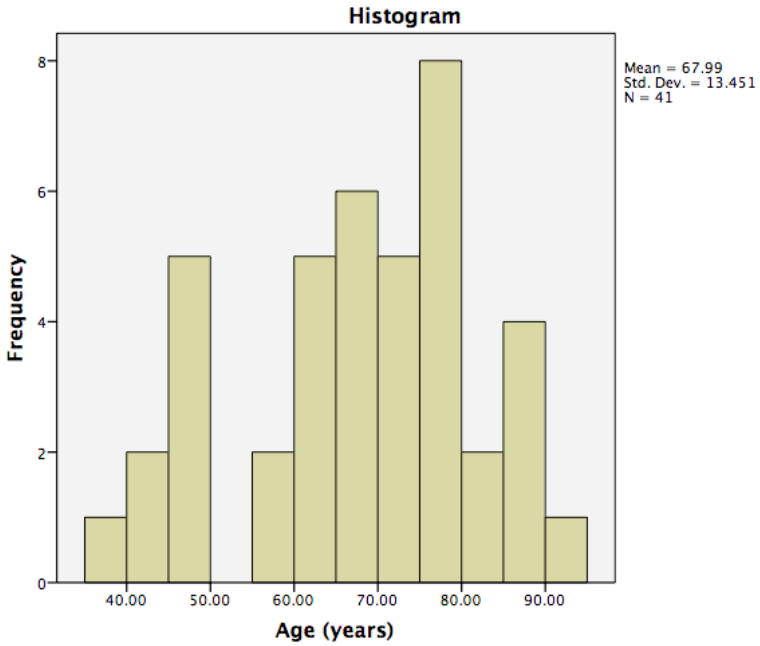

Figure 11: Age distribution.

6.4 Results

The mean overall initial Hb level was 115.8 g/dl (SD=20.4), with 10 patients being referred with a diagnosis of IDA (24.4%). Amongst those referred with IDA, the mean Hb was 95.3 g/dl, significantly lower than those not identified as IDA who had a mean Hb of 124.3 g/dl (*t*-test, P<0.0001). Twenty patients were referred with rectal bleeding (48.8%), 26 (63.4%) with a change in CIBH, 6 (14.6%) with weight loss and 1 (2.4%) with a significant family history. Hb levels were significantly lower in patients referred with rectal bleeding (107.1 g/dl with and 126.3 g/dl without, *P*=0.002). There was no significant difference in Hb level between patients according to CIBH (119.6 g/dl with CIBH and 111.5 g/dl without, *P*=0.245) or abdominal mass (109.3 g/dl with a mass, 118.3 g/dl without, *P*=0.323).

All patients had a confirmed diagnosis of CRC: 10 right-sided (24.4%), 31 left-sided (75.6%) – comprised of 16 patients with left-sided colonic (39.0%) and 15 with rectal tumours (36.6%). Overall, there were 26 patients with a colonic primary (63.4%) and 15 (36.6%) with a rectal primary.

There were 8 patients (19.5%) with stage I disease, 12 stage II (29.3%), 15 Stage III (36.6%) and 6 stage IV (14.6%). There was an uneven distribution of patients by stage, with relatively more patients with stage II and III disease than I and IV disease (see Table 9 and Figure 12, *P*=0.003), which was not altered significantly between those with colonic or rectal primaries (Table 6, *P*=0.212). The presence of LVI was available in 36 patients of whom 25 had no LVI (69.4%) and 11 with LVI (30.6%).

Stage	Colonic (N, %)	Rectal (N, %)	Total (N, %)
Stage I	4 (15.4%)	4 (26.7%)	8 (19.5%)
Stage II	9 (34.6%)	3 (20.0%)	12 (29.3%)
Stage III	10 (38.5%)	5 (33.3%)	15 (36.6%)
Stage IV	3 (11.5%)	3 (20.0%)	6 (14.6%)
Total	26 (100.0%)	15 (100.0%)	41 (100.0%)

Table 9: Cancer stage.

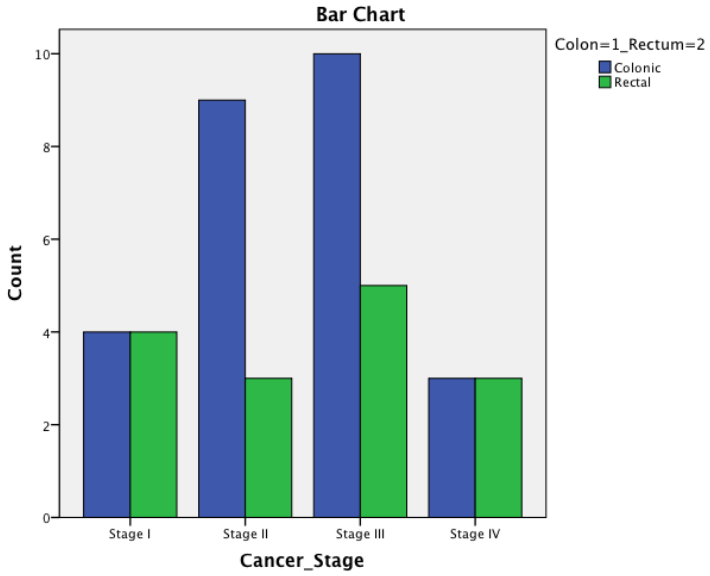

Figure 12: Cancer Stage.

Of the 41 patients, *KRAS* status was available in 40 of whom 28 were wild type (wt, 70.0%), 7 mutation 12 (17.5%) and 5 mutation 13 (12.5%). *BRAF* was available in 24 patients of whom 14 were wt (82.5%) and 3 with V600E mutation (17.6%).

There was no significant correlation in presentation Hb levels and eventual disease staging (*P*>0.05 for all associations). Patients with right-sided tumours were found to have a lower Hb level than patients with either left-sided (*P=0.017)* or rectal tumours *(P=0.009)*. Hb levels were also significantly lower in patients with the *BRAF* V600E mutation (*P=0.009*). *KRAS* status was not found to be associated with Hb levels (*P>0.05)* (T=Table 10). LVI status as well, was not found to be linked with Hb levels (*P>0.05).*

Feature	Hb (g/dl)
Total	115.8
Stage I	116.0
Stage II	114.6
Stage III	120.6
Stage IV	105.7
Right-sided	98.6*$^{+}$
Left-sided	120.3*
Rectal	122.3^{+}
KRAS wt	116.6
KRAS 12	108.1
KRAS 13	120.4
BRAF wt	123.5*
BRAF 600	89.0*
LVI-negative	115.0
LVI-positive	109.7

Table 10: Relationship of Hb level to clinical features (ANOVA associations)

*Disease Stage P>0.05 for all associations with Hb levels; Primary location *P=0.017 (Right-sided vs Left sided tumours' Hb levels), $^{+}$P=0.009 (Right-sided vs Rectal tumours' Hb levels); KRAS status P>0.05 for all associations with Hb levels; BRAF status *P=0.009; LVI status P=0.456.*

6.5 Discussion

There has been a long discussion about clinical significance of *BRAF* V600E mutation in colorectal cancer [146, 177, 180]. Recent studies conclude that *BRAF* V600E mutation is associated with poorer prognosis [122, 146] and support its role in the response to chemotherapy [76, 144, 171]. More specifically, Mao *et al*. [95] state that *BRAF* V600E is associated with resistance to anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibodies in patients with metastatic CRC and wild type *KRAS*. Phipps *et al.* [172, 181] conclude that *BRAF* mutational prognostic value can vary, depending on patient and tumour characteristics. Kristen *et al.* [182] highlight the significance of *BRAF* in terms of prognostic value, as well as a new CRC therapy target.

However, there is also emerging data, which liaise *BRAF* V600E with MSI-high tumours [167]. In those cases, there is still a negative effect of the V600E mutation in overall survival [113], though some other researcher still doubt it [72, 116, 117, 183] and support that *BRAF* V600E has a negative prognostic effect only in microsatellite stable (MSS) tumours.

BRAF V600E is generally linked in the literature with certain clinicopathological features, *i.e.* female sex and mucinous differentiation [166, 167, 184, 185]. There is a well-defined relationship between V600E mutation and generally poorer prognosis; however, no convincing explanation has been given yet.

Our study concludes that there is a well-defined relationship between significantly lower Hb levels and V600E mutant neoplasms comparing to the wild type samples. There is no doubt that this could be attributed to the fact that all those patients present with right-sided tumours and also by the fact that all *BRAF* V600E specimens were advanced stage tumours (T3b and above). However, this observation could alter the significance of the presence of IDA in the

clinical investigations pathway by demonstrating that IDA may signify a higher risk cancer. This may well affect the choice of treatment and would imply to look for *BRAF* mutation in unexplained IDA, where colorectal cancer is suspected. Of note is the fact that we could not identify any statistical significance between Hb levels and disease stage (I-IV).

The main question that this study raises is in what stage of the carcinogenesis pathway does *BRAF* interfere. According to the literature, most of the *BRAF* V600E specimens appear to be in advanced stage [93, 177, 184]. It is, thus, quite important to establish in which time point *BRAF* interferes as it appears that this happens on a mature stage in the pathway. Taking into consideration the latter, it is quite important to interpret the potential link between IDA at presentation and *BRAF* V600E.

A recent promising study [185] suggests a multitarget stool DNA test with faecal immunochemical test (FIT) as a noninvasive diagnostic technique for CRC screening. The sensitivity for detecting CRC was 92.3% with DNA testing and 73.8% with FIT detecting *versus* 42.4% with DNA testing and 23.8% with FIT in advanced precancerous lesions. This test does not include *BRAF*. The main question is whether there would be of clinical importance to incorporate *BRAF* screening in these tests, especially in those cases where IDA is noted at presentation. This would help in increasing the sensitivity of these methods and IDA could be used as an alarm point where advanced cancer with mutant *BRAF* is suspected. In terms of precancerous lesions, again, our question regarding the stage of the carcinogenesis that *BRAF* interferes, could be a crucial point in order to improve the sensitivity of this method, as well as stratifying the risk of potential progression to cancer. In other words, if we knew the exact point where V600E mutation appears, then, we would be able to tell if a precancerous lesion has a potential higher risk of progression to cancer.

Moreover, we tried to liaise potential clinical presentation features with the molecular status of *KRAS* and *BRAF*, which are thought to play a significant role in prognosis of CRC, as well as

response to neoadjuvant chemotherapy. The main question was whether there would be any unique clinical presentation or histopathology features, which could potentially be liaised with a known mutation of *KRAS* or *BRAF*.

With regards to *KRAS* mutational status, there was no significant relationship between disease stage and *KRAS* status ($P>0.05$). This keeps with what already exists in literature, as *KRAS* is not an established prognostic marker [177]. It is well known that in many centres around the world, *KRAS* can be used as a predictive tool to identify whether a patient would benefit from anti-EGFR chemotherapy agents [70, 76, 119, 134]. Nevertheless, despite its well known predictive value, there is no conclusion whether *KRAS* status could be used as a prognostic tool, with the vast majority of researchers doubting its significance in terms of defining disease-free survival [70, 119]. An interesting point from or results is that there was no direct relationship between Hb levels or clinical presentation and *KRAS* status ($P>0.05$). This could be easily explained by the fact that CRC pathogenesis is quite complex and a single gene is difficult to be liaised with specific macroscopic presentation features unless there is an established strong relationship between certain histopathology characteristics and molecular status. Similarly, there was no defined statistical relationship between LVI and *KRAS* mutational status ($P>0.05$), which could be attributed again to its controversial prognostic value.

KRAS mutations tend to appear in earlier stages of the carcinogenesis pathway [166]. Contrary to *BRAF* V600 E neoplasms, where there is an established relationship with proximal location, *KRAS* mutant tumours tend to have a bimodal distribution in the proximal-distal axis and tend to appear more proximally and to the caecum [85].

On the other hand, *BRAF* V600E mutation is well linked with proximally located tumours [184]. On our sample, even though it is quite small, there was a well-defined relationship between right-sided neoplasms and V600E mutation.

Nevertheless, there was no defined statistical relationship between LVI and *BRAF* status ($P>0.05$) and this is difficult to comment based on the limitations of our sample. Positive LVI is linked with a slightly lower Hb level comparing to LVI negative (109.7 g/dl *vs.* 115.1 g/dl).

In conclusion, given the complexity of the molecular pathways described, as well as the fact that many molecular agents are interfering one each other, it is quite difficult to establish a clear picture for unique partial impact of every gene in the CRC pathogenesis. Our study concludes to raise a question regarding the stage of the carcinogenesis pathway where *BRAF* appears to be mutant. Also, it is quite important to consider whether IDA could be used as a potential marker of advanced cancer and, given its relationship with proximal tumours and subsequently with *BRAF* V600E mutation, this could be used as a starting point to question whether V600E mutation could be incorporated in DNA screening methods that are recently been released. This would be indisputably useful in cases where IDA could potentially be a sign of more advanced disease.

7 KRAS Mutant Status, p16 and β-catenin Expression may Predict Local Recurrence in Patients who Underwent Transanal Endoscopic Microsurgery (TEMS) for Stage I Rectal Cancer. [PMID: 27798894]

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Keywords: Colorectal cancer, biomarkers, transanal endoscopic microsurgery, KRAS, recurrence.

Authors' contributions: MS has contributed to collecting and analyzing the data and drafting the manuscript. SP is the senior author of the study who conceived the study, interpreted the data and edited the manuscript and has operated the series of these patients. SP is the primary MD(Res) supervisor of MS. IB and AH have contributed to the editing of the manuscript, as well as providing feedback on the interpretation of the data. IB is the second supervisor of MS's MD(Res). JM and SDC have contributed to the molecular analysis of the specimens. JM is the lead of the Advanced Diagnostics Lab of KCH working in close collaboration with SDC who is the Lead Consultant Histopathologist.

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Conflicts of interest: The Authors declare no conflict of interest.

Short title: Sideris et al. KRAS, β-catenin and p16 significance in early rectal cancer

Cover Letter: attached

Informed consent statement: We confirm that all the authors have approved this manuscript and contributed as stated below.

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Type of Study: Original Research

7.1 Abstract.

Background: Transanal endoscopic microsurgery (TEMS) is emerging as an alternative treatment for rectal cancer Stage I. There remains a risk of local recurrence. Aim: To study the effect of biomarkers in local recurrence for Stage I rectal cancer following TEMS plus or minus radiotherapy. Materials and Methods: This is a case control study where we compared 10 early rectal cancers that had recurred, with 19 cases with no recurrence, total 29 patients (age=28.25-86.87, mean age=67.92 years, SD=14.91, Male, N=18, Female, N=11). All patients underwent TEMS for radiological Stage I rectal cancer (yT1N0M0 or yT2N0M0) established with combination of magnetic resonance imaging (MRI) and ERUS. We prospectively collected all data on tumour histology, morphological features, as well as follow-up parameters. Molecular analysis was performed to identify their status on BRAF, KRAS, p16 O^6-methylguanine-DNA methyltransferase (MGMT) and β-catenin. Results: From 29 specimens analysed, 19 were KRAS wild type (65.9%) and 10 mutant (34.5%). Recurrence of the tumour was noted in 10 cases (34.5%) from which 60% were pT1 (N=6) and 40% pT2 (N=4). There was a statistically significant association between KRAS mutant status and local recurrence (N=6, p=0.037). P16 expression greater than 5% (mean=10.8%, min=0, max=95) is linked with earlier recurrence within 11.70 months (N=7, p=0.004). Membranous β-catenin expression (N=12, 48%) was also related with KRAS mutant status (p=0.006) but not with survival (p>0.05). BRAF gene was found wild type in all cases tested (N=23). Conclusion: KRAS/p16/β-catenin could be used as a combined biomarker for prediction of local recurrence and stratification of the risk for further surgery.

7.2 Introduction

The method of local excision, which has gained wider acceptance, recently, in early rectal cancer, is transanal endoscopic microsurgery (TEMS). TEMS generally offers advantages in operative access and oncological clearance over transanal resection (TAR) but recently a number of similar logic techniques with various rectal ports for endoscopic excision of rectal tumours has been invented. Those methods are collectively named transanal minimal invasive surgery (TAMIS) and for oncological purposes they share the same features of local excision as TEMS. [43, 51-53, 55, 56, 61, 64, 65, 71, 149, 186-188]

TEMS is a minimally invasive technique that was introduced by Buess in the early 1980's [51]. Through the new rectoscope with 3D binocular optic and the endoscopic instruments, it offers better access to proximal lesions with lower margin positivity and fragmentation and magnification of the operative field [51, 189]. TEMS is a safe procedure that offers low complications' rate and peri-operative morbidity (10.7%) [55]. There have been multiple studies to suggest that TEMS is the operation of choice for rectal adenomas [186], retrorectal and submucosal rectal lesions [53]. Furthermore, TEMS offers the advantage of not damaging the anorectal function [52].

TEMS is indicated as a curative treatment for malignant neoplasms that are histologically confirmed as pT1 sm1 carcinomas, whereas T1 sm2-3 and T2 lesions are still under question [56]. A number of studies have shown that TEMS can have comparable results with radical surgery [56, 64, 186, 190] for rectal cancer.

There has been a concern about oncologic outcomes following TEMS [41]. Some studies support that there is potentially a higher risk of local recurrence rate with TEMS [56, 149, 191]. Efforts have been made to classify risk with morphological and histological criteria with better patients' selection [43, 52, 56, 61, 186]; however, the risk stratification remains imperfect.

Molecular biomarkers have been used in prognosis of colorectal cancer (CRC) in general and rectal cancer seems to follow the same genetic phenotype [192]. So far, the data in the literature, which link biomarkers and oncologic outcomes from TEMS, are limited.

Several biomarkers are being studied in CRC. Nevertheless, rectal cancer may have a specific profile of biomarkers, which is different to the rest of colonic cancer [193]. Kohonen-Corish *et al*. [193] went through and analysed a cohort of 381 rectal cancer specimens. The conclusion was that they identified a more aggressive subgroup arising from the *KRAS*-p16 pathway. This was explained by the fact that p16 deficiency and *KRAS* mutant status did promote carcinogenesis through the loss of oncogene-induced senescence. Therefore, p16 and *KRAS* could potentially be used as a prognostic biomarker in rectal cancer, which is against what is thought to be the case in sporadic colonic cancer.

We thus, aimed to identify whether any of the biomarkers *KRAS*, *BRAF*, p16. MGMT, β-catenin could be used as a prognostic factor to predict recurrence in early rectal cancer following local excision (TEMS).

7.3 Materials and Methods

Our institution performs TEMS for early rectal cancer after discussion at the Multi-Disciplinary Cancer meeting (MDT) according to UK national guidelines. All cases in this study have been operated by the same surgeon (senior author, SP). We prospectively collected data on histological parameters of each lesion *i.e*. stage, differentiation, location, margins of resection and dysplasia where applicable. Preoperative staging of the tumours has been performed with magnetic resonance imaging (MRI) of the rectum and endorectal ultrasound (EUS), which are proven to have high diagnostic accuracy when combined [194-197]. We have an established follow-up protocol for these patients with an intensive 5-year surveillance consisting of 6 monthly endoscopy, carcinoembryonic antigen (CEA), MRI scan and computed tomography (CT) scan for 3 years, which is altered to annual surveillance in years 4 and 5 (Table 11).

We selected 10 patients who had recurrence after TEMS for early rectal cancer and we compared them in a case control study with 19 similar patients without recurrence. This was to assure we could compare the status of several biomarkers in non-recurrent *vs*. recurrent lesions. Eighteen patients (62.1%) were male and 37.8% were female (N=11). Mean age at the stage of diagnosis, was 67.93 years (min=28.25, max=86.87). All the specimens were Stage I rectal cancer. Eighteen were pT1 (62.1%) and 11 pT2 (37.9%). There were no differences between the two groups.

Radiological staging has been performed both before and after radiotherapy. Neoadjuvant or adjuvant radiotherapy decision was based on fitness criteria, as well as patient choice. The option of further radical (completion) surgery has been offered in selected individualised cases as an option but was declined by the patients.

Biomarker analysis. All biomarkers were assessed on formalin-fixed, paraffin -embedded (FFPE) samples. Beta-catenin, mismatch repairs (MMRs), O^6-methylguanine-DNA

methyltransferase (MGMT) and p16 assays were performed using immunohistochemistry (IHC). Four-μM sections of the tumour were cut on to coated slides and IHC was performed using standardised protocols for each antibody. The final step was visualisation of antigen-antibody complexes by the addition of the chromogen, diaminobenzidine. The slides were then assessed (by SDC and JM) and scored for percentage of positive tumour cells and protein location (Figure 13-16).

KRAS mutation analysis was performed on the same tumour samples. Haematoxylin and eosin (H&E) stained sections of the tumour were assessed and marked for tumour content and the tumour was then macrodissected using serial, unstained section from the non-tumour components, allowing for enrichment of tumour cells. DNA was then extracted from these samples using standardised protocols and quantified by Qubit analysis. Polymerase chain reaction (PCR) using primers either side of the regions of interest, codons 12 and 13 and codon 61, was performed. After immobilisation of the resulting amplicons, single stranded DNA was prepared and the sequencing primer for each region was annealed. The samples were then analysed on a Qiagen Q24 pyrosequencer (QIAGEN Group®, PyroMark®, Pyrosequencing®, 1061692 02/2010 © 2010 QIAGEN, all rights reserved) and the resulting sequence was analysed using the appropriate software (PyroMark Q24 Software, 1/2009 ©). The mutation status of each tumour was reported according to standard protocols (by SDC and JM).

Appropriate positive and negative controls were included for all assays.

Statistical analysis. Statistical analysis of our results was performed using IBM SPSS for Macintosh version 22 (IBM Corp., Armonk, NY, USA). We have performed bivariate correlations (Pearson's and Spearman's rho, as well as Chi-square associations) to identify any potential links between follow-up parameters and *KRAS*, *BRAF*, p16, β-catenin or MGMT.

7.4 Results

The mean overall follow-up period was 32.83 months (SD=23.02, min=12.96, max=126.9). From 29 patients, 19 did not have recurrence (65.5%), whereas 10 did have local recurrence of the rectal lesion (34.5%). The mean recurrence time was 13.04 months (min=4.11, max= 42.28) (Table 11).

From 29 patients, 79.3% (N=23) did not require further surgery, whereas 21.6% did (N=6). Twenty-five patients warranted TEMS procedure once (86%), whereas 1 patient required TEMS procedure twice (3.4%), 1 three times (3.4%) and 2 four times (6.8%). Decision to proceed with multiple TEMS was based on the fact that patients were unfit for radical surgery. Three patients (10.3%) received neoadjuvant radiotherapy (N=1 was pT1 and N=2 pT2), whereas 89.7% (N=26) did not receive. N=9 received further course of radiotherapy, based on the histology findings of unsuspected cancer and individualised decision to proceed with radiotherapy was made by the Multidisciplinary Team meeting. Patients underwent short course radiotherapy; the regimen was 45-50Gy according to the local guidelines. All patients who had repeat TEMS surgery were elderly or unfit for major surgery and, therefore, the procedure was palliative (Table 11).

All the 29 specimens were Stage I from which 62.1% were pT1N0 (N=18) and 37.9% were pT2N0 (N=11). This is based on radiological criteria. Completion of staging was performed with the rest of preoperative work up, including EUS, clinical assessment, CT abdomen-pelvis-thorax (TAP) and MRI of the pelvis and rectum. Almost one third (31.0%) did not have confirmed clear resection margins (N=9), whereas in the rest 69% (N=20), the margins were confirmed to be clear. The remaining tumours had margins obscured by the energy device artefact (Harmonic Ace and electro cautery) and were declared uncertain. There were no positive margins. N=19 (65.5%) of the lesions were located in the lower rectum, 20.7% (N=6)

in the mid rectum and 13.4% (N=4) in the upper rectum. Two (6.9%) were poorly differentiated adenocarcinomas, whereas 62.1% (N=18) were moderate differentiated and 31% (N=9) were well differentiated. The mean circumference of the tumour was 40.80% (min= 20%, max=75%). Greater than 33% circumference was found to be linked with mutant *KRAS* (p=0.00) (Table 12).

Nineteen of the specimens were *KRAS* wild type (65.5%) whereas 34.5% (N=10) were *KRAS* mutant (codon 12, 13 or 61). From the available *BRAF* results, 23 of the specimens were analysed and none was found to be positive for V600E mutation (wild type). From the MGMT point of view, 91% (N=23) were positive (preserved), whereas 8% (N=2) were negative (non-preserved). Four patients did not have available MGMT status (Table 12).

From 29 specimens, 25 had available β-catenin status. Almost half (48%) were found to express membranous (m) status of β-catenin (N=12) (Figure 13), whereas 28% was found to express membranous and 30% nucleus (n) (N=7) (Figure 14), 10.3% m+50% n (N=3) and 10.3% only nucleus (N=3).All *KRAS* mutant tumours were linked with membranous β-catenin (N=8, p=0.009), whereas wild-type *KRAS* were spread between β-catenin m (N=3, 23.1%), m+30%n (N=7, 53.8%), m+50%n (N=3, 15.2%) and 100%n (N=1, 7.6%). Also, membranous β-catenin was linked with more circumferential configuration of the tumour (p=0.028) but not with survival (p>0.05) (figure 18).

From our primary analysis, there was an association between mutant *KRAS* and local recurrence (p=0.037). From 10 specimens, which recurred, 60% were mutant for *KRAS* (N=6) and 40% wild type (N=4). Also, from 19 specimens with no recurrence, 79% (N=15) were wild type and 21% (N=4) *KRAS* mutant (Table 13). There was no association between recurrence and p16 or β-catenin or MGMT status (p>0.05). Furthermore, local recurrence was not found to be associated with MRI pT stage, (p>0.05) or other morphological features of the tumour, including height, location, circumference and orientation (p>0.05). Local recurrence was not

found to be associated with histological features of the specimens, *i.e*. pT stage, clear margins, dysplasia or differentiation ($p>0.05$). Moreover, there was no association between recurrence and neoadjuvant radiotherapy ($p=0.123$) (Table 12).

The overall mass was calculated from the dimensions documented on the histology report (size 1 in mm x size 2 in mm x size 3 in mm). The overall mean mass of the specimens was 13229,967 mm^3 min=0.00 mm^3, max=159.250,00 mm^3, SD=28.706,9. The mean mass of the tumours that recurred was 25.776,00 mm^3 (min= 3200,00 mm^3 max= 159.250,00 mm^3, SD=47.375,34) compared to 6626,78 mm^3 (min=00 mm^3, max=17.940,00 mm^3, SD=5121,69) for those that did not recur. There was a statistical significant relationship between the mass and recurrence of the lesions ($p=0.028$).

KRAS was found to be associated with β-catenin status ($p=0.009$) and, more specifically, mutant *KRAS* tends to be linked with membranous (m) β-catenin (N=12, 41.9%). Also, *KRAS* mutant status is associated with the increased number of total TEMS ($p=0.02$). All wild type *KRAS* (N=19) specimens have undergone a single operation, whereas 11.1% (N=1) of mutant had times 2 TEMS, 11.1% (N=1) times 3 and 22.2% (N=2) times 4. What is more, cancer-related death was noted in 6.9% (N=2) and was linked with *KRAS* mutant status ($p=0.045$). Discovery of unsuspected cancer was noted in 41.3% (N=12) and linked with mutant *KRAS* (N=7, $p=0.023$). However, there was no link between *KRAS* and dysplasia or differentiation of the specimen or clear margins or mass or pT stage or orientation of the specimen ($p>0.05$ for all associations, Table 12).

Mean p16 expression was 10.8% (min=0.0%, max=75%). P16 expression more than 5% was linked with recurrence within the first 11.70 months (N=7, $p=0.04$) (Figure 19), though no other histological, morphological or phenotype association was identified ($p>0.05$) (Table 12) (Figure 15-17).

Finally, MGMT status was found preserved in 92.0% (N=23) and non-preserved (negative) in 8.0% (N=2). All patients with MGMT negative results were aged above 80 years at the time of diagnosis (p=0.024) and pT2 (p=0.030). There was no other association noted between MGMT and morphological, histological or follow-up parameters (p>0.05) (Table 12).

7.5 Discussion

There has long been an effort to link molecular biomarkers with prognosis in colorectal cancer but it still remains a poorly understood field. From our data, there seems to be a link between mutant *KRAS* and the risk of local recurrence of early rectal cancer after endoscopic local excision surgery TEMS. This is a previously unreported finding. We discuss here whether there may be a sound biological basis of this finding.

Kirstein rat sarcoma viral sarcoma oncogene (*KRAS*), is a proto-oncogene involved in cellular response to extracellular stimuli, *i.e.* mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase / v-akt murine thymoma viral oncogene pathways (PI3K/AKT) [69, 113]. Therefore, *KRAS* mutations can potentially lead to activation of downstream signaling pathways, *i.e.* MAPK and PI3K/AKT. The latter is thought to lead to a higher resistance to anti-epidermal growth factor receptor (EGFR) inhibitors and, hence, this could be the etiology for increased resistance to anti-EGFR chemotherapy agents [198, 199].

KRAS mutations in codons 12, 13 and 61 are the most popular and tend to occur in 30-40% of CRCs. However, there have been noted 85 different *KRAS* mutations, many of which refer to a specific cancer pathway [56, 119].

So far, the prognostic and predictive value of *KRAS* gene in CRC has been debated, though it still remains controversial. There have been studies, which mainly support *KRAS* predictive value in response to anti-EGFR chemotherapy agents [198]. More specifically, wild type *KRAS* is thought to be linked with better response to anti-EGFR agents, *i.e.* cetuximab, and this could be attributed to the relevant molecular mechanism described in the previous paragraph [199]. Nevertheless, there are emerging data that *KRAS* predictive value in response to anti-EGFR inhibitors is controversial when *BRAF* gene appears to be mutant (V600E mutation) [93, 142, 179].

On the contrary, the prognostic value of *KRAS* in CRC remains equivocal. Most of the studies are inconclusive [70, 119] but there are some that associate *KRAS* mutant status with poorer prognosis [172].

Our study concludes that mutant *KRAS* may create a higher risk of local recurrence of early rectal cancer following TEMS. In our data, there is no association between local recurrence and pT stage or any other histopathological features ($p>0.05$); however, we need to keep in mind this is a small study not powered enough to test all possible factors. A recent study [85] supports that the frequency of *KRAS* mutant colorectal carcinomas is 35-40%, where in our case is 34.5%.

KRAS is also linked with some morphological features of the tumors and this can support its impact on the recurrence [85]. Given the fact that a change in the genotype can directly reflect on the tumor phenotype, we can support that *KRAS* is linked with more circumferential configuration ($p=0.000$) and height of the lesion ($p=0.030$), as well as bigger mass ($p=0.029$).

BRAF V600E seems to be another valid prognostic biomarker in CRC. There is a link between *BRAF* V600E status and worse prognosis [122, 146] and *BRAF* V600E and more advanced cancer, being mostly present in right-sided tumours [93, 177]. As discussed before, *BRAF* V600E has been found to interfere with *KRAS* occasionally. There are studies supporting that mutant *BRAF* status can affect response to anti-EGFR chemotherapy agents even in the presence of wild type *KRAS* [76, 95]. All our specimens were *BRAF* wild type, where tested, and this could be explained by both the location, as well as earlier stage.

Another interesting finding in our data, is that p16 and MGMT are associated with older age ($p=0.01$ and $p=0.024$, respectively). _ENREF_43Methylation of CpG islands (CpG island methylator phenotype (CIMP) and, specifically, O(6)-methylguanine-DNA methyltransferase (MGMT) gene promoter methylation) has been studied extensively in CRC [72, 80, 116] and seems to have a recognised impact on the carcinogenesis pathway, though there is no consensus

about its prognostic value [200]. Loss of MGMT expression is found in 30-40% of metastatic CRC and results in the inability of the alkyl base to be removed from the methylated guanine and, hence, preservation of the DNA is not achieved [200].

With regards to the prognostic value of p16, there are studies correlating the methylation of p16 promoter with several clinicopathological features of CRC [93, 155, 201], *i.e.* TNM stage (p=0.006), lymph node metastasis (p=0.002), histologic grade (p<0.001), Dukes stage (p=0.002), tumour size(p=0.041) and location (p<0.001) Also, another study [202] shows that p16 methylation is found more in the serum of metastatic CRC patients. On our study, there is a link between p16 expression >5% and recurrence within 11.5 months (p=0.04). Furthermore, a recent cohort study [203] concludes that patients with advanced CRC, p16, human mutL homolog 1 (hMLH1) and MGMT methylation are associated with higher risk of recurrence, compared to patients with preserved unmethylated promoters.

Another interesting recent study [204] concludes that approximately 70% of *KRAS*-positive tumors are thought to have a CIMP characterised by aberrant methylation of the DNA of multiple genes, including p14, p15, p16. Therefore, this could be used as a link between *KRAS* status and p16 expression, especially in different stages of the cancer pathway.

In terms of the β-catenin significance, there seems to be a link between *KRAS* mutant status and membranous β-catenin (N=8, p=0.09). This is fairly interesting as it connects two different pathways of the carcinogenesis pathway together. Beta-catenin seems to belong to a different signalling pathway (wnt/β-catenin) and its significance in CRC remains equivocal. Beta-catenin is a pivotal molecule involved in intercellular adhesion and some other oncogenic and developmental processes [205]. The same study links the up-regulation of β-catenin with the confrontation of tumour cells in the host microenvironment, whilst in metastatic process. However, its role in CRC remains unclear. In our study, membranous β-catenin is linked with higher circumference of the tumor (p=0.028, Figure 18). This supports again a joint role with

mutant *KRAS* status as the latter is linked with higher circumference as well. An interesting study [176] in lung cancer concludes that combinational activation of *KRAS* and wnt/β-catenin pathway leads to a significant increase on the lung tumour formation and, therefore, worse prognosis effect.

With regards to the decision for further neoadjuvant radiotherapy, no relationship was identified between the decision for neoadjuvant radiotherapy and our potential biomarkers, *i.e.* *KRAS*, *BRAF*, MGMT, β-cateninand p16 ($p>0.05$ for all associations).

In summary, our study has limitations of size, which does not allow clarification of all the contributing factors in recurrence. As recurrence of early rectal cancer after local excision is rare, with average numbers around 10%, it is not easy to form a much larger study than this. However, we consider our findings adequate to propose that *KRAS*, p16 expression and β-catenin status should be looked further as a potential combined biomarker system to assess the risk of local recurrence. Mutant *KRAS* is found to be associated more with local recurrence and with unsuspected cancer in the specimen ($p=0.023$) (when the initial tumor was thought dysplastic). Membranous β-catenin tends to be linked with mutant *KRAS* as well. P16 expression is associated with earlier recurrence, something that could re-direct the focus of the 5-year postoperative surveillance protocol if confirmed.

In conclusion, the biomarkers *KRAS*/p16/β-catenin could be used as a combined biomarker for prediction of local recurrence and stratification of the risk for further surgery, as well as to stimulate further larger studies to confirm these findings and increase our understanding of the factors causing recurrence after local excision of early rectal cancer.

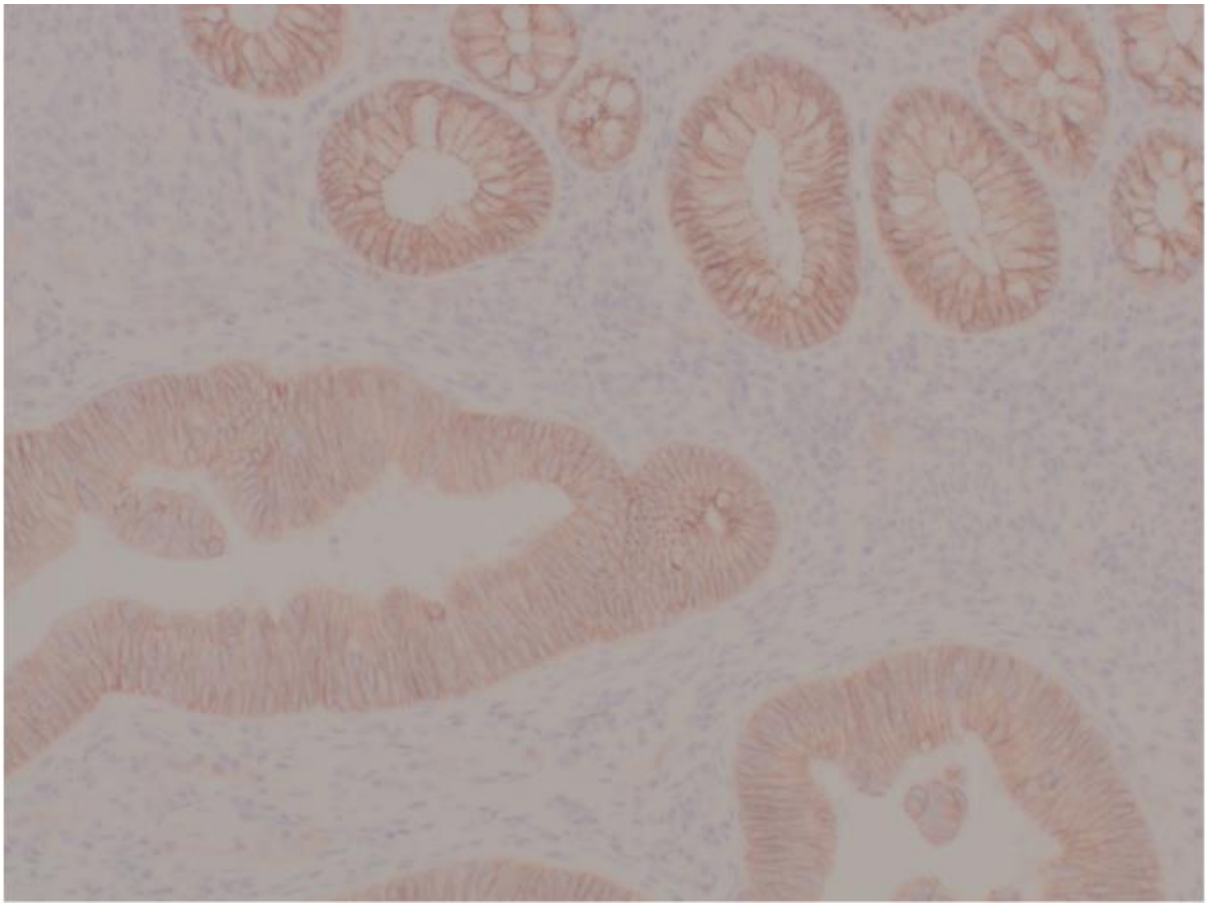

Figure 13: Beta-catenin membranous

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Original photo from KCH lab (J. Moorhead).

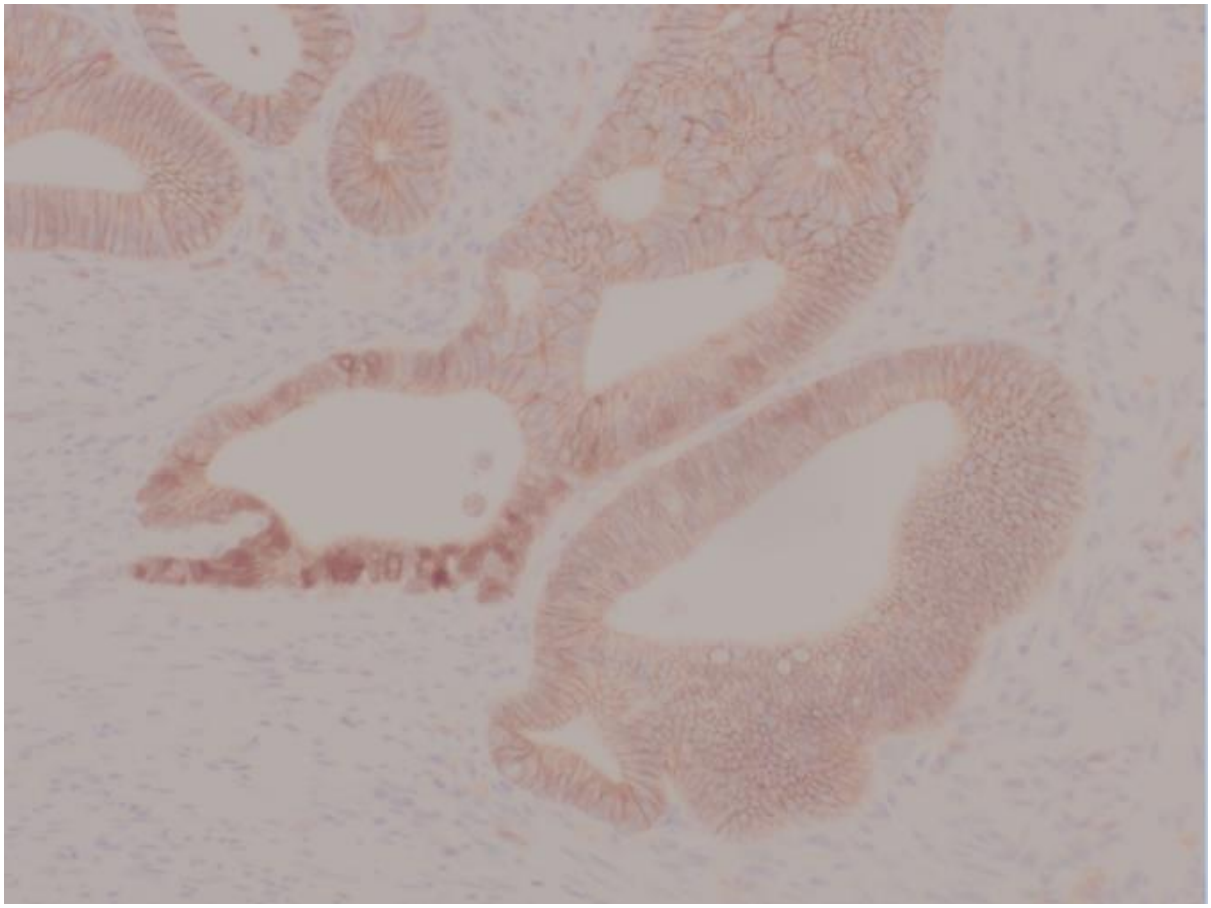

Figure 14: Beta-catenin membranous and nuclear

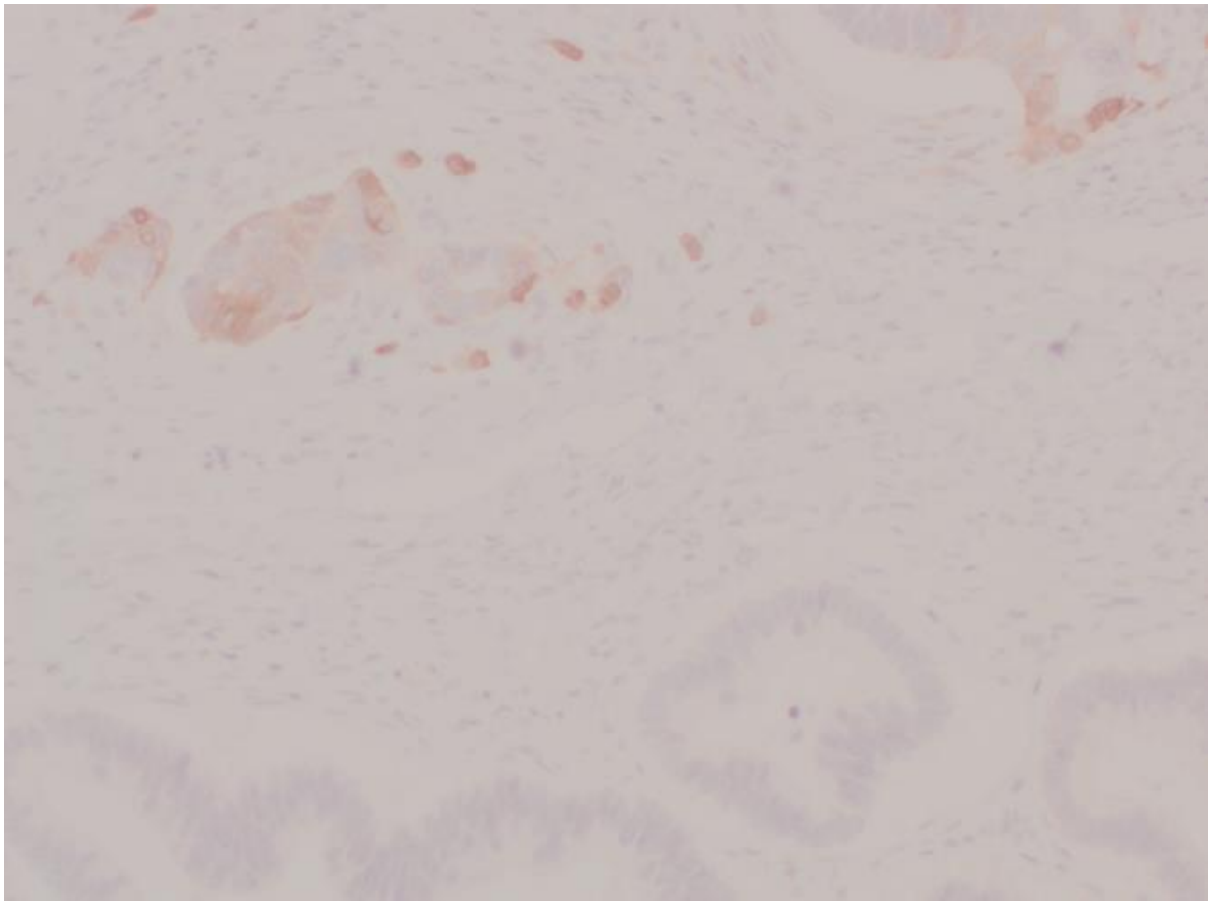

Figure 15: P16 expression.

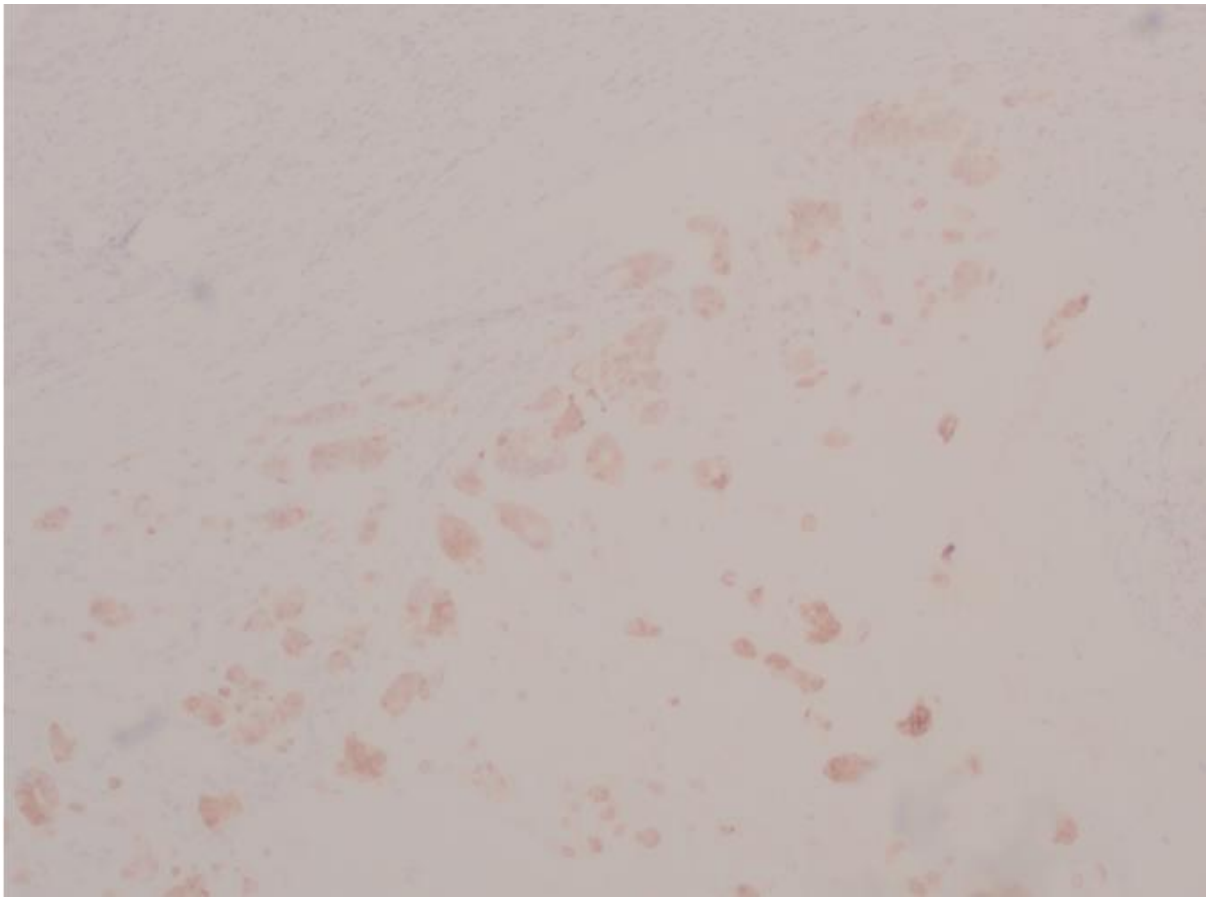

Figure 16: P16 expression (higher %).

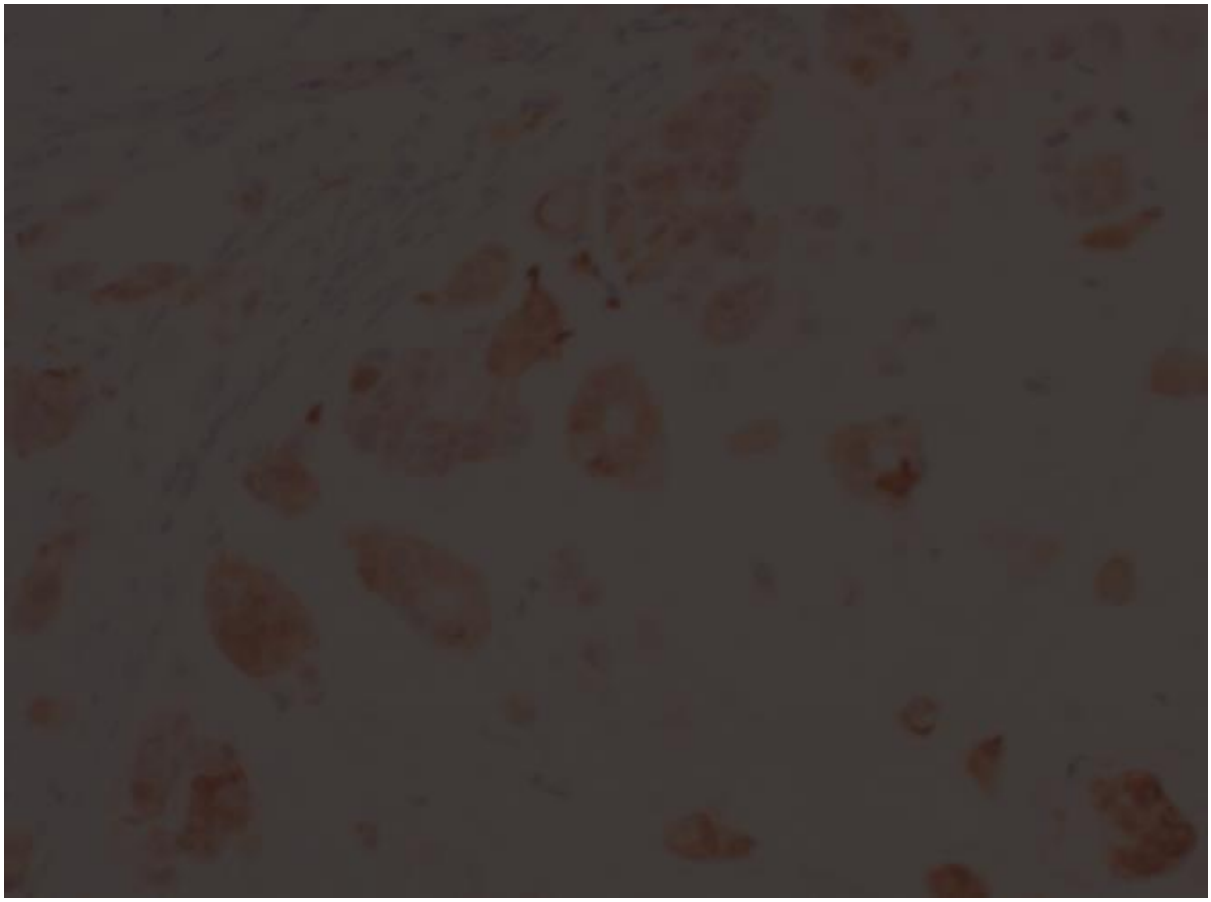

Figure 17: P16 expression

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Patients' characteristics (Table 11)				
Age (Diagnosis)	Minimum=28.25, Maximum= 86.87, Mean age=67.92 years			
pT stage	pT1N0, N=18 (62.1%)		pT2N0, N=11 (37.9%)	
Recurrence	YES, N=10 (34.5%)		NO, N=19 (65.5%)	
Follow-up	Mean follow-up=32.83months, SD=23.02, min=12.96, max=126.9			
Mean recurrence	Minimum=4.11 months, Maximum=42.28, Mean= 13.04 Months			
Clear margins	YES, N=N=20 (69%)		Inconclusive, N=10 (31%)	
Location	Lower rectum, N=19 (65.5%)	Mid rectum, N=6, (20.7%)		Upper rectum, N=4 (13.4%)
Differentiation	Well dif., N=9 (31%)	Moderately Dif., N=18 (62.1%)		Poorly Dif., N=2, (6.9%)
TEMS (NOP)	Times x1, N=25 (86%)	Times x2, N=1 (3.4%)	Times x3, N=3 (3.4%)	Times x4, N=2 (6.8%)
Mean circumference	Minimum= 20%, Maximum=75%, mean=40.80%			
Volume/Mass	Minimum =0 mm^3, Maximum=159.250,00 mm^3, Mean volume=13.229.97 mm^3			
Neoadjuvant radiotherapy	YES, N=3 (10.3%)		NO, N=26 (89.7%)	
Adjuvant radiotherapy	YES, N=9 (31%)		NO, N=20 (69%)	

KRAS	Wild type, N=19 (65.5%)		Mutant, N=10 (34.5%)	
BRAF	All analysed specimens (N=23) were *BRAF* wild type			
MGMT	Preserved, N=23 (91%)		Non-preserved, N=2 (9%)	
β-catenin	m, N=12 (48%)	m+30n%, N=7 (30%)	m+50n%, N=3, (10.3%)	n, N=3, (10.3%)
P16	Minimum= 0%, Maximum=75%, Mean expression =10.8%			

Table 11: Patients' characteristics, specimens' morphology, biomarkers' profile

NOP, number of procedures.

.

		KRAS		Recurrence		Other association
	F	**Wild type**	**Mutant**	**YES**	**NO**	
P16	O-75% Mean= 10.8%	0-40% *p*=0.258	0-80% *p*=0.258	0-30% *p*=0.761	0-80% *p*=0.761	> 5% → recurrence in 10 months **(*p*=0.04)**
β-catenin	NA	any	Membranous N=8 ***p*=0.009**	m&m+30n *p*=0.724	Any *p*=0.724	Higher circumference (*p*=0.028)

MGMT	P(N=23) NP(N=2)	P(N=15),NP(N=1) *p*=0.683	P(N=8),NP(N=1) *p*=0.683	P(N=8),NP(N=1) , *p*=0.683	P(N=15), NP(N=1) , *p*=0.683	All NP were 80 years old and above ***p*=0.024** and pT2, ***p*=0.030**
Differentiation	W(N=9) M(N=18) P(N=2)	W(N=7) M(N=11) P(N=2) *p*=0.774	W(N=3) M(N=7) P(N=0) *p*=0.774	W(4) M(6) P(0) *p*=0.317	W(N=5) M(N=12) P(N=2) *P*=0.317	NA
Dysplasia	H(N=5) M(N=1) NA(N=23)	H(N=2) M(N=0) NA(N=17) *p*=0.057	H(N=3) M(N=1) NA(N=6) *p*=0.057	H(N=3) M(N=0) NA(N=7) *p*=0.445	H(N=2) M(N=1) NA(N=16) *p*=0.445	NA
Location	L(N=19) M(N=6) U(N=4)	L(N=12) M(N=4) U(N=3) *p*=0.177	L(N=8) *p*=0.177	L(N=7), M(N=2), U(N=1) *p*=0.690	L(N=12), M(N=4), U(N=3) *p*=0.690	NA
Clear margins	Y(N=20) N(N=9)	Y(N=14) N(N=5) *p*=0.467	Y(N=6) N(N=4) *p*=0.467	Y(N=6) N(N=4) *p*=0.467	Y(N=14) N(N=5) *p*=0.467	NA

pT stage	pT1(N=18) pT2(N=11)	pT1(N=11) pT2(N=8) *p*=0.540	pT1(N=7) pT2(N=3) *p*=0.540	pT1(N=6) pT2(N=4) *p*=0.873	pT1(N=12) pT2(N=6) *p*=0.873	NA
MRI pT stage	pT0(N=3) pT1(N=7) pT2(N=9) pT3(N=4)	pT0(N=3) PT1(N=4) pT2(N=6) pT3(N=2) *p*=0.390	pT0(N=0) pT1(N=3) pT2(N=3) pT2(N=2) *p*=0.390	pT0(N=0) pT1(N=3) pT2(N=3) pT3(N=2) *p*=0.390	pT0(N=3) pT1(N=4) pT2(N=6) pT3(N=2) *p*=0.390	NA
Circumference	20-75% Mean=40.80%	20-50% ***p*=0.00**	30-80% ***p*=0.00**	20-80% *p*=0172	20-70% *p*=0.172	NA
Age	28.25-86.87 Mean=67.93	Any *p*=0.109	Any above 60 *p*=0.109	Any above 30 *P*=0.338	Any *p*=0.338	NA
Mass	0-159.250 Mean=13229.97	>17940 *p*=0.073	>159.250 *p*=0.073	>17940 ***p*=0.028**	>159.250 ***p*=0.028**	NA

Sex	M(N=18) F(N=11)	M(N=9) F(N=10) ***p*=0.024**	M(N=9) F(N=1) ***p*=0.024**	M(N=8) F(N=2) *p*=0.160	M(N=10) F(N=9) *p*=0.160	NA
Cancer-related death	Y(N=2) N(N=27)	Y(N=0) N(N=19) ***p*=0.045**	Y(N=2) N(N=8) ***p*=0.045**	Y(N=2) N(N=8) ***p*=0.045**	Y(N=0) N(N=19) ***p*=0.045**	NA

Table 12: Significant associations

KRAS status	Recurrence		Significance	
	YES	NO	Total	
Wild type	N=4 (13.8%)	N=15 (51.7%)	N=19(65.5%)	*p*=0.037
Mutant	N=6 (20.7%)	N=4 (13.8%)	N=10(34.5%	*p*=0.037
Total	N=10 (34.5%)	N=19 (65.5%)	N=29(100%)	

Table 13: KRAS and local recurrence

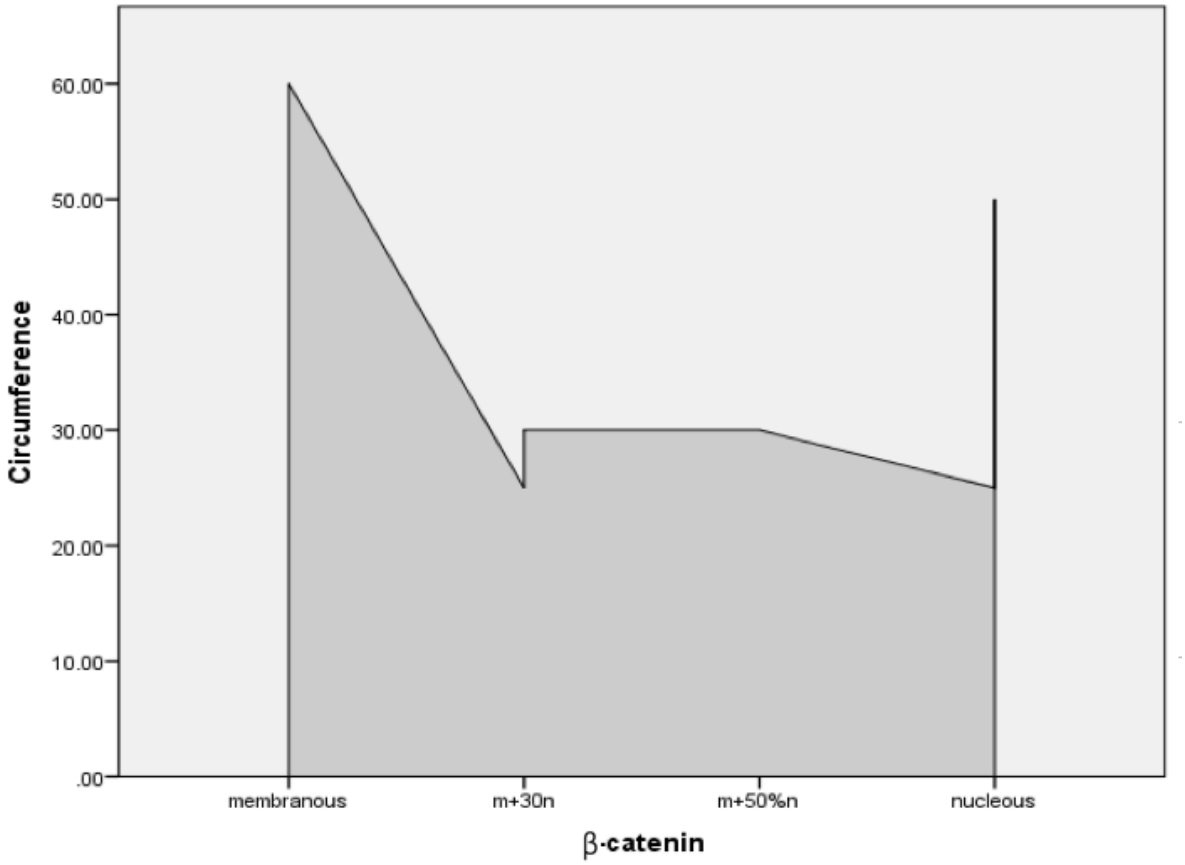

Figure 18: Circumference vs. β-catenin expression

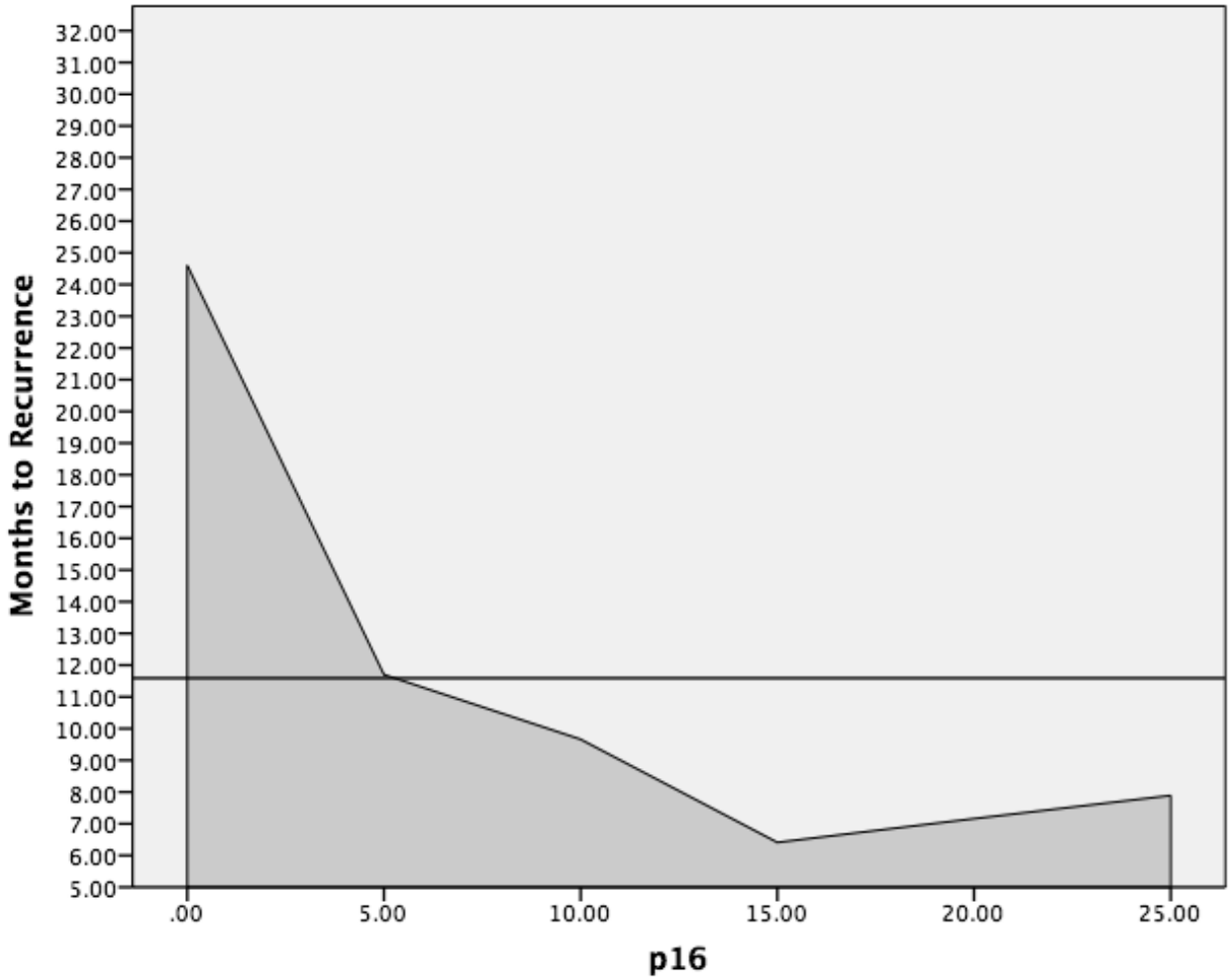

Figure 19: P16 in relationship with time of recurrence

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8 KRAS Mutant Status May Be Associated with Distant Recurrence in Early Stage Rectal Cancer. [PMID: 28314302]

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Keywords: Molecular biomarkers, early rectal cancer, *KRAS*, recurrence.

Short Running Title: KRAS Mutant Prognostic Value in Early Rectal Cancer

8.1 Abstract.

Background/Aim**:** Total mesorectal excision combined with neo-adjuvant chemoradiotherary (CRT) and adjuvant chemotherapy, has been the standard treatment of locally advanced rectal cancer (LARC). Although TNM (Tumor, Node, Metastasis) Classification for Malignant Tumors is still the cornerstone in rectal cancer staging, there has been an effort to identify molecular biomarkers with additional prognostic or predictive value. Materials and Methods: We retrospectively analyzed molecular biomarkers on prospectively collected histology specimens and clinical data from a cohort of 135 consecutive Rectal Cancer Cases who underwent radical excision in a tertiary center between 2011-2014 (M=87, F=48, age: 22-89, mean=64,67, SD=13.40). Radiological, histopathological, molecular staging, treatment stratification by the multidisciplinary team (MDT), as well as prognostic outcome data were compared with various biomarkers including KRAS, BRAF, p16, b-catenin, MSI, MMR and MGMT. Results: The mean follow-up was 39.21 months (range=5-83 months, SD=21.34). 28 cases were Stage I (20.9%), n=30 Stage II (22.4%), n=45 Stage III (33.6%) and n=31 Stage IV (23.1%). Forty specimens were KRAS-mutant (mt) (37.4%) while n=67 (62.6%) wild type (wt). KRAS mt status was associated with female sex (n=20, p=0.021) and older age (69.62 vs. 62.27, p=0.005). Stage I Early Cancer Subgroup analysis showed that KRAS mt status is associated with distant recurrence of disease (n=4, p=0.045). Conclusion: KRAS mt status may affect the prognosis of early rectal cancer, as this is linked with distant recurrence.

8.2 Introduction

Treatments for rectal cancer have evolved significantly during the last decade [78]. Bowel cancer screening is starting to diagnose tumors at an earlier stage [206, 207]. This has ~~significantly~~ notably improved survival and made minimally invasive treatments more feasible. Neo-adjuvant Chemo radiotherapy (CRT), followed by total mesorectal excision (TME) and adjuvant chemotherapy, remains the gold standard in the treatment of Locally advanced rectal cancer (LARC) [196]. LARC generally refers to Stage III rectal cancer, and it represents 70% of the rectal cancers on presentation [208].

Recent developments in the study of oncogenes and biomarkers, have raised hopes ~~that they may~~ of possible assistance in prognosis and treatment choices [70, 80, 119]. Molecular biomarkers are grossly divided into prognostic and predictive. ~~The former~~ Prognostic biomarkers refer to those molecules, that could potentially hold important information on life expectancy post diagnosis +/- treatment of disease, whereas ~~the latter~~ predictive biomarkers are used to predict response to a treatment plan [69, 70]. Although there is progress in the understanding of the significance of those biomarkers, the complexity and diversity of carcinogenesis pathways in Colorectal Cancer (CRC), makes it challenging to identify their impact on prognosis, and potential implications to treatment choice [86].

KRAS is a proto oncogene which seems to be a well-recognized predictive biomarker in CRC [70]. Most of the studies comment on KRAS ~~value~~ expression as a tool to predict response to anti-EGF chemotherapy, though its prognostic value is still ~~equivocal~~ ambiguous [209]. *BRAF* V600E mutation has also been well studied and ~~it~~ seems to be associated with proximal tumors as well as poorer prognosis [103]. Microsatellite instability (MSI) is a part of the molecular phenotype of CRC, and hence has been widely studied in both hereditary ~~as well as~~ and

sporadic CRC [78]. Methylation products seem to play an increasingly significant role as well. Currently, many studies look at hypermethylation of CpG islands which are generally located at the promoters of various genes, the expression of which can affect CRC outcomes [72, 82, 116, 202, 210]. For instance, p16 and b-catenin protein expression as well as MMR or MGMT preservation are currently tested routinely in our institution, for completion of the molecular staging of the CRC cases. Our aim in this study is to examine the significance of certain molecular biomarkers including KRAS, BRAF, MMR, MGMT, p16, MSI, b-catenin in the prediction of recurrence and survival.

8.3 Materials and Methods

Samples. One hundred and thirty-five rectal cancer cases with prospective collection of clinical and pathological data (as part of the UK National Bowel Cancer Audit) were used for this study. Analysis of biomarkers was performed on stored tumor specimens. Our center offers treatment options to patients, based on the multidisciplinary team MDT approach. Intense follow-up on a 6-month basis, according to local guidelines. This protocol includes CT Abdomen, Thorax and Pelvis, colonoscopy, CEA, and occasionally EUS or MRI of the abdomen. Data on demographics, radiological, histopathology, molecular as well as follow up outcomes are prospectively collected as part of the UK National Bowel Cancer Audit.

Molecular analysis: All biomarkers were assessed on formalin fixed, paraffin embedded (FFPE) samples. B-catenin, MMRs, MGMT and p16 assays were performed using immunohistochemical analysis (IHC). Four-μM sections of the tumor were cut on to coated slides and IHC was performed using standardised protocols for each antibody. The final step was visualization of antigen-antibody complexes by the addition of the chromogen, 3-3'-diaminobenzidine (DAB). The slides were then assessed (by SDC and JM) and scored for percentage of positive tumor cells and protein location.

KRAS mutation analysis was performed on the same tumor samples. H&E stained sections of the tumor were assessed and marked for tumor content and the tumor was then macro-dissected using serial, unstained section from the non-tumor components, allowing for enrichment of tumor cells. DNA was then extracted from these samples using standardized protocols and quantified by Qubit analysis. PCR using primers either side of the regions of interest, codons

12(35G>A, 35G>T, 34G>T) and 13(38G>A) and codon 61 (182A>T), was performed. After immobilization of the resulting amplicons, single stranded DNA was prepared and the sequencing primer for each region was annealed. The samples were then analyzed on Quigen Q24 pyrosequencer© and the resulting sequence was analyzed using the appropriate Qiagen software (Version 2.07). The mutation status of each tumor was reported according to standard protocols (by SDC and JM).

Appropriate positive and negative controls were included for all assays.

Statistical analysis: Statistical analysis of our results was performed using IBM SPSS for Macintosh version 22 (IBM Corp. Armonk, NY, USA). Univariate correlations (Pearson's and Spearman's rho as well as Chi-square crosstabs) were used to identify any potential links between various parameters and KRAS, BRAF, p16, b-catenin or MGMT. Independent *t*-test associations were used to compare means in different groups. Statistical significant level was set at p=0.05.

8.4 Results

One hundred and thirty-five patients with confirmed rectal cancer were identified in our cohort (mean age at diagnosis was 64.67 years old, range=22-89 years, SD=13.32). Eighty-seven (64.4%) patients were male, and 48 (35.6%) were female. The mean follow-up was 39.21 months (range=5-83, SD=21.34). 21 (16.3%) were ASA grade I, 72 (55.8%) ASA grade II, 35 (27.1%) ASA grade III and 1 was ASA grade IV (0.8%) (Table 14).

Preoperative staging (MDT Stage): *Twenty-eight* were Stage I (20.9%), n=30 were Stage II (22.4%), n=45 were Stage III (33.6%) and n=31 were Stage IV (23.1%). Stage was defined during the MDT meeting based on either radiology or pre-op biopsy if available (Table 15).

Neoadjuvant treatment: *Seventy-one* (52.6%) did not receive any neo adjuvant treatment and went straight to surgery. N=10 (7.4%) received neo-adjuvant radiotherapy only prior to surgery, from whom n=3 (30%) were radiological Stage II and n=7 (70%) were radiological Stage III. N=38 (29%) received neo adjuvant chemo radiotherapy, from whom n=20 (52.6%) were radiological Stage IV, n=15 (39.5%) Stage III, n=2 (5.3%) Stage II and n=1 (2.6%) Stage I. N=12 received neo adjuvant chemotherapy, from whom n=9 (75%) were radiological Stage IV, n=2 (16.7%) Stage III and n=1 (8.3%) Stage II. All decisions were based on MDT preoperative staging (Table 14).

Pathological staging (postoperatively): All the 135 cases were adenocarcinomas. N=23 (17.4%) specimens were well differentiated, n=94 (69.6%) were moderately differentiated, and n=8 (5.9%) were poorly differentiated. N=10 (7.3%) were non-specified (Table 15).

N=32 (23.7%) were Stage I, n=38 (28.1%) Stage II, n=33 (24.4%) Stage III and n=31 (23%) Stage IV. This was because some of the patients were offered down-staging neo adjuvant therapy. N=9 were pT1 (7.1%), n=34 were pT2 (26.6%), n=69 were pT3 (53.9%), n=16 (12.5%) were pT4. N=78 were pN0 (61.9%), n=26 were pN1 (20.6%), n=22 were pN2 (17.5%). In n=53 (67.1%) there was LVI negative, whereas, in n=26 (32.9%) it was LVI positive.

In n=12 there was Perineural Invasion noted (PNI), whereas in n=67 there was negative PNI. In the rest of the cases there was no PNI or LVI status documented on the biopsy report.

Finally, in n=77 the margins were clear (71.3%), whereas in n=31 (28.7%) histology was inconclusive. Regarding the rest n=27 of the cases there was no documentation on the biopsy report. The mean number of the lymph nodes (LN) was 15.08 (range=0-41, SD= 7.13), and the mean number of positive LN was 1.83 (0 - 24, SD=3.73). The mean ratio LN+ve/LN total was 0.107 (range=0-1, SD=0.202).

Molecular staging: Molecular analysis was performed in theatre specimens. All were BRAF wild-type (n=65). There was only n=1 MSI-H and n=95 MSS (MSI Stable). The mean p16 expression was 12.63% (min=0, max=70%, SD=15.33). n=63 (94%) were MGMT preserved whereas n=4 (6.0%) were MGMT non-preserved. In n=94 cases MMR was preserved, whereas in n=1 MMR was not preserved. With regards to beta-catenin, in n=27 it was Membranous (M), in n=22 (34.8%) M and focal Nucleus (N), in n=10 (15.9%) mixed M+N and in n=4 (6.3%) N (Table 16). Associations between molecular biomarkers and outcomes are shown in Table 17.

Adjuvant treatment: Seventy-six (58%) patients did not receive any adjuvant treatment, while n=49 received adjuvant chemotherapy (37.4%) based on the pathological staging. From those 49 patients, n=4 were Stage I (8.2%), n=10 (20.4%) Stage II, n=18 (36.7%) were Stage III and n=17 (34.7%) were Stage IV. n=2 received some additional radiotherapy (1.5%) from whom, n=1 was Stage I and n=1 Stage IV, as they both were not fit for further surgery. N=4 (3.1%) had adjuvant chemo radiotherapy, from whom n=1 was Stage I, n=1 was Stage III and n=2 were Stage IV (Table 14).

Outcomes: *One hundred and two* cancer cases (75.6%) did not recur (Table 18). In n=6 there was local recurrence (4.4%), whereas n=27 (20%) suffered distant recurrence. With regards to local recurrence n=1 was pT1, n=1 pT2, n=3 pT3 and n=1 pT4. n=3 were pN0, n=1 pN1 and n=2 pN2. n=5 were pM0 and n=1 pM1. Overall n=1 was Stage I, n=1 Stage II, n=3 Stage III and n=1 Stage IV. Concerning distant recurrence, n=6 were Stage I (22.2%), n=5 (18.5%) Stage II, n=4 (14.8%) Stage III and n=12 (44.4%) Stage IV. The mean time to recurrence was 18.66 months (min=17.00, max=22, SD=2.84) for local recurrence and 10.22 months (range=2-32, SD=8.63) for distant recurrence. In n=13 (9.6%) there was Cancer-related death. The mean time of Cancer-related death was 26.39 months (range=0-52, SD=17.60). No other association between KRAS status and histopathological features (pNI, +ve LN/LN, pTNM, LVI), follow up parameters (recurrence, adjuvant/neo-adjuvant chemo/radiotherapy) or radiological staging (uTNM), was noted (p>0.05) (Table 17).

In the only MSI-H specimen, there was positive PNI (p=0.020), and similarly for the n=1 case with non-preserved MMR expression (p=0.021). ~~What is more~~ Furthermore, the mean age of cases with non-preserved MGMT was higher, compared to preserved MGMT (83.50 *vs.* 66.53,

p=0.005). No other significant association for MGMT status was noted (*p*>0.05 for all associations) (Table 17).

Otherwise, recurrence was positively associated with positiveve LN [1.09 (for no recurrence) *vs.* 1.33 (for local recurrence) *vs.* 4.68 (for distant recurrence), *p*< 0.001], as well as the ratio +ve LN/LN [0.066 (no recurrence), *vs.* 0.722 (for local recurrence) *vs.* 0.278 (for distant recurrence) *p*<0.001] and pM Stage (*p*=0.005), as well as overall Stage of the disease as defined by the MDT (*p*=0.022). However, positiveve LN / LN ratio, seems not to be associated directly with any of the molecular biomarkers (*p*>0.05 for all associations) (Table 17).

Stage I Cases - Subgroup analysis results: In the second part of our analysis we examined biomarkers in stage I rectal cancer exclusively. N=32 cases were identified, and n=20 were male (62.5%) whereas n=12 were female (37.5%). The mean age was 65.15 (min=40, max=85, SD=11.97). N=3 (9.4%) had neo-adjuvant chemo radiotherapy prior to surgery, n=1 (3.1%) chemotherapy and n=1 (3.1%) radiotherapy. N=1 received adjuvant radiotherapy (3.1%), n=4 chemotherapy (12.5%) (Table 19). N=8 were pT1 (27.6%), n=21 were pT2 (65.6%). N=7 (21.9%) were well-differentiated adenocarcinomas, n=20 (62.5%) were moderately (70.0%), n=1 (3.6%) poorly and n=4 were unspecified. The mean ratio +ve LN/ LN was 0.375 (min=0, max=1, SD=0.20). n=17 had no LVI +ve (94.4%), whereas n=1 had positive LVI (5.6%). All the specimens were pNI negative (n=20). N=21 had complete margins (87.5%), whereas n=3 had inconclusive margins (12.5%), and in the rest n=8 there was no documentation (Table 19). The mean follow-up was 35.25 months (min=11, max=72, SD=20.046), and there was no cancer related death. In n=22 of the cases there was no recurrence (78.6%), n=1 was local recurrence (3.1%) and n=6 distant (18.8%). The mean recurrence time was 11.5 months (3 - 25.00, SD=8.57) (Table 20).

With regards to molecular status, n=12 were KRAS wt (52.2%), and n=11 (47.8%) were KRAS mt. MSI was stable in all cases (n=20) and MMR expression was preserved as well (n=20). MGMT status was preserved in n=15 cases and non-preserved in n=1 from the specimens that were analyzed. The mean p16 expression was 16.85% (min=1.00, max=50.0, SD=14.40). b-catenin was M in 53.3% of the cases (n=8), focal N and mainly M in 26.7% (n=4) and mixed N + M in 20.0% (n=3) (Table 20).

KRAS-mutant status seems to be associated with distant recurrence (n=5, p=0.045). No other significant association was noted (p > 0.05). Membranous b-catenin seems to be associated with distant recurrence (n=3 distant recurrences / n=8 M expression), though this did not reach statistical significance due to low numbers (p=0.098). However, no association was noted between b-catenin status and any other histopathological, radiological or follow outcomes (p>0.05 for all associations). No other significant associations with regards to molecular biomarkers were noted (Table 20).

There were no cancer-related deaths, therefore we cannot comment whether there is any link between KRAS and cancer related deaths (Table VII). Distant recurrence of disease positively associated with the number +ve LN (p=0.049) as well as increasing ratio +LN/LN (p=0.05) as expected. There was no association between KRAS and +ve LN or the the ratio +ve LN/LN overall (p>0.05) (Table 20).

8.5 Discussion

The pathway of carcinogenesis in CRC is a complex multifactorial process [86]. Sporadic CRC pathway can result from the down regulation of tumor suppressor genes, ~~or~~ activation of oncogenes, or from various discrepancies in the mismatch repair genes (MSI), or even from chromosomal losses (CIN) [80, 86, 119], [211].

KRAS is a proto-oncogene, that is involved in the *mitogen-activated protein kinase* (MAPK) and *phosphoinositide-3-kinase/v-akt murine thymoma viral* oncogene pathways (PI3K/AKT). More specifically KRAS, regulates cellular response to extracellular stimuli. Its mutant status can lead to downstream activation of the latter pathways (MAPK, PI3K/AKT)[69, 199]. This is the potential mechanism, through which, mt KRAS can result in resistance against anti-EGFR chemotherapy agents [199].

The incidence of KRAS mutations ~~is~~ comprises around 35-40% of CRC cases, which is confirmed by our study as well (37.4%) [85]. Point-mutations in codons 12,13 and 61 are ~~supposed~~ considered to be the most common, though approximately 85 different mutations have been identified [119]. Rosty *et al.* [85] managed to define various distinct clinicopathological features, which are associated with KRAS mutant status *i.e.* mucinous differentiation, proximal location, certain MSI status, MGMT methylation, and presence of contiguous polyp ($p<0.05$ for all associations). The same study notes that mt KRAS is associated with female sex, which was the case for our study as well ($p=0.021$). Notably, in our study, there was also a significant association between older age in mt KRAS compared to wt (69.62 *vs.* 62.26, $p=0.005$), which could be attributed to the fact that carcinogenesis is a lengthy process, accumulating distinct mutations.

Despite the predictive value of KRAS in better response to anti-EGFR chemotherapy [198], its prognostic value remains debatable [70, 119], and there is not enough evidence with regards to KRAS prognostic value in purely rectal cancer. In our cohort of rectal tumors, KRAS wild type is linked with cancer related death for any stage (n=10, p=0.033). Colorectal cancer follows complex and variable pathways, and in this case, it seems that KRAS may not be involved in the carcinogenesis process.

The most important finding of our study is that KRAS-mutant status seems to be associated with distant recurrence in Stage I rectal cancer (p=0.045). This seems to support the assumption that early cancer recurrence can represent the reflection of a specific carcinogenesis pathway where KRAS plays an important role, and it is involved at early stages. Similar findings were reported in one of our recently published study [104], where KRAS is linked with local recurrence in Stage I Rectal Cancer following Transanal Endoscopic Microsurgery (TEMS).

On the other hand, if KRAS is found mutant in more advanced stages of rectal cancer, this may be a consequence of a different molecular pathway, where prognosis is defined by other molecular parameters. No other association was found between KRAS status and distinct histopathological features (pTNM, positive LN / LN, PNI, LVI, p>0.05 for all associations) or radiological parameters or survival outcomes (p>0.05).

In a recent meta-analysis in 2017, Tosi et al, included 1833 patients [212] and concluded that, KRAS mutant status is negatively associated with overall survival (OS) and relapse-free survival (RFS) in patient who underwent liver resection for metastatic CRC. Brudvik et al [213], report similar findings in their meta-analysis of 1809 cases, published in 2015. Jones et al [66] evaluated 392 cases of advanced and recurrent colorectal cancer from a UK Centre, and, concluded that codon 12 mutations are independently associated with worse overall survival after diagnosis. Similar findings are reported from another US study [88, 89], where KRAS mutations, seem to be independent risk factors for worse OS. Moreover, the same team,

reported in another study[87], that KRAS codon 13 mutation, is associated with higher risk for overall extrahepatic or lung specific recurrence.

BRAF V600E mutation seems to be another valid biomarker in CRC. Its presence has been associated with poorer prognosis [122, 146], and is deemed more as feature of right-sided, advanced CRC [103, 177, 201]. In our study, BRAF was found wt and this is explained by its association with proximal colorectal cancer. Recently, there have been studies, which associate BRAF V600E mutation with resistance to anti-GFR inhibitors, regardless the presence of wt KRAS [76, 95].

Methylation of CpG islands, and especially, O(6)-methylguanine DNA methyltransferase (MGMT) gene promoter methylation has been deemed to play a role in the CRC pathway, however, consensus regarding its prognostic value, has yet to be reached [72, 80, 200]. MGMT is a DNA repair enzyme, codified at locus 10*q*26, which removes alkyl groups from the O6 position, and this leads to irreversible inactivation of the enzyme [200, 214]. Hence, loss of MGMT expression, via methylation of the CpG islands of its promoter, could be reflected, with alteration of normal DNA [210, 215, 216]. Non-preserved MGMT expression is found in 30-40% of metastatic colorectal cancer [200].

In our study, non-preserved MGMT expression was associated with higher age (66.53 *vs.* 83.50, p=0.005), which should, again, raise a question, whether older age would be deemed as an independent factor to worse prognosis, exclusively based on the accumulation of more genetic events, resulting in a distinct molecular phenotype.

B-catenin is considered an essential molecule, that belongs to Wnt-signaling pathway. Wnt/b-catenin pathway is primarily involved in the regulation of oncogenic processes, as well as intracellular adhesion [205]. There is still little consensus on the role of b-catenin in CRC pathway. A recent study found that KRAS and Wnt pathway may interact in lung cancer [176]. In our study, there was a trend towards an association between M expression of b-catenin and

distant recurrence (n= 6 p=0.092). In Stage I subgroup analysis, there is a similar trend (n=3, p=0.096). However, these were only trends and none of them reach statistical significant values, therefore their interpretation value is limited. There was no association between b-catenin and neo-adjuvant/adjuvant therapy, and any other histopathological features (pTNM, +ve LN/LN, LVI, PNI, p>0.05 for all associations).

With regards to p16 expression, there is a recent meta-analysis [155], which associates various clinico-pathological features with the deregulation of its promoter via methylation. In our cohort, there is a trend towards higher p16 expression in M b-catenin (21.66 *vs.* 10.25 *vs.* 5.00, p=0.028). Interestingly, there was no association between the status of any of the biomarkers studied in our cohort (KRAS, BRAF, MMR, MGMT, p16, b-catenin) and the response to neo-adjuvant therapy (p>0.05) or other pathological features.

Finally, we recognize the limitations of this study which is predominantly the relatively small sample size, that does not allow to use multivariate statistics. However, our conclusions can raise interesting questions for further research.

8.6 Conclusion

Carcinogenesis of CRC is a complex and multifactorial event, in which various molecular events interfere. We found that when KRAS is mutant in early rectal cancer, this fact may be linked with higher chance of distant recurrence. This is an interesting finding that should be further examined with greater amount of research in hope that it may constitute an additional prognostic factor for early rectal cancer.

8.7 Tables.

Age	Mean Age= 64.67, [22, 89], SD=13.32 (years)			
Sex	Male 87(64.4%)		Female 48(35.6%)	
ASA	Grade I 21(16.3%)	Grade II 72(55.8%)	Grade III 35(27.1%)	Grade IV 1(0.8%)
Follow up	Mean= 39.07 [5, 83], SD=21.3 (months)			
Treatment				
Surgery	Laparoscopic AP Resection 87(64.9%)	Open AP resection 41(30.6%)	Conversion to Open 5(3.7%)	Endoscopic Treatment 1(0.7%)
Complications	n=78(57.8%) uncomplicated		n=57(42.2%) minor complication	
	Received		Received	
Neo-adjuvant treament	Chemotherapy	Radiotherapy	None	ChemoRadio
	n=12 (8.9%)	n=10 (7.4%)	n=71 (52.6%)	n=38 (29%)
	Received		Received	
Adjuvant Treatment	Chemotherapy	Radiotherapy	None	ChemoRadio
	n=49 (37.4%)	n=2 (1.5%)	n=76 (58%)	n=4 (3.1%)

Table 14: Patient's demographics and treatment.

	Pre-operative Stage (I-IV)			
Stage	I 28(20.9%)	II 30(22.4%)	III 45(33.6%)	IV 31(23.1%)
Post-operative Stage (I-IV)				
Stage	I 32(23.7%)	II 38 (28.1%)	III 33(24.4%)	31 (23.0%)
Histology	135(100%) adenocarcinomas			
Differentiation	23(17.4%) Well	94(69.6%) moderately	8(5.9%) poorly	
pT Stage	T1 9(7.1%)	T2 34(26.6%)	T3 69(53.9%)	T4 16(12.5%)
pN Stage	N1 78(61.9%)	N2 26(20.6%)	N2 22(17.5%)	
pM Stage	M0 95 (77.3%)		M1 28(22.7%)	
LN	Mean 15.08 [0.0 -41], SD=7.13			
LN +ve	Mean 1.83 [0.0- 24], SD=3.73			
LN +ve /LN	Mean 0.107, [0-1], SD= 0.2020			
CRM	Clear 77 (71.3%)		Inconclusive 31(28.7%)	
LVI	Positive 26 (32.9%)		negative 53 (67.1%)	
PNI	Positive 12 (15.2%)		Negative 67 (84.8%)	

Table 15: Preoperative and pathological staging.

Molecular Staging (I-IV)				
KRAS	Mutant n=40 (37.4%)		Wild type n=67 (62.6%)	
BRAF	Wild type n=65 (100%)			
MSI	MSI-H n=1 (1%)		Stable n=95 (99%)	
P16	Mean 12.63% [0-70] SD=15.33			
MGMT	Non-preserved 4(6.0%)		Preserved 63 (94%)	
MMR	Non-preserved 1(1.1%)		Preserved 94 (98.6%)	
B-catenin	M 27(42.9%)	M + focal N 22(34.9%)	Mixed N+M 10(15.9%)	N 4(6.3%)

Table 16: Molecular staging

M, Membranous; N=nucleic.

KRAS Status (I-IV)	*Vs*. Feature			Total/*p* value
	No Recurrence	Local	Distant	
Wild type	47	3	17	67
Mutant	32	1	7	40 p=0.526
	No PNI	PNI		
Wild Type	33	8		41
Mutant	20	2		22 *p*=0.242
	No LVI	LVI		
Wild Type	25	17		42
Mutant	22	5		27 *p*=0.017
	Male	Female		
Wild Type	48	19		67
Mutant	20	20		40, *p*=0.021
	+LN / LN			
Wild Type	0.095			
Mutant	0.1141			*p*=0.658
	Mean Age			
Wild Type	62.26			
Mutant	69.62			*p*=0.005

B-Catenin Status (I-IV)	*Vs.* feature			Total/p-value
	No Recurrence	Local	Distant	
M	20	1	6	27
M + focal N	19	0	3	22
M + N	9	1	0	10
N	4	0	0	4 *p*=0.092
	No PNI	PNI		
M	16	3		19
M + focal N	6	2		8
M + N	5	0		5
N	1	0		1 *p*=0.504
	No LVI	LVI		
M	16	6		22
M + focal N	13	3		16
M + N	8	1		9
N	2	2		4 *p*=0.952
	Male	Female		
M	16	11		27
M + focal N	15	7		22
M + N	6	4		10
N	4	0		4 *p*=0.339

Table 17: Biomarkers vs. outcomes.

Outcomes				
	Yes		No	
Recurrence	33(24.4%)		102(75.6%)	
	Local n=6 (4.4%)	Distant n=27 (20%)		
Mean Time Recurrence	14.8months [2-54], SD=13.44			
Cancer Related Deaths	n=13 (9.6%)		Mean Time 26.39 [0-53], SD=17.6	

Table 18: Outcomes (Recurrence, Cancer Related Deaths).

Patient's Demographics Stage I (n=32)			
Age	Mean Age= 65.15, [40, 85], SD=11.97 (years)		
Sex	Male 20(65.2%)	Female 12(37.5%)	
Follow up	Mean= 35.25 [11, 72], SD=20.046 (months)		
Treatment			
	Received		
Neo-adjuvant Chemotherapy n=1 (3.1%)	Neo-adjuvant n=3 Chemoradiotherapy n=3(13.8%)	Neo-adjuvant Radiotherapy n=3(9.3%)	None n=25

	Pathological Stage **I**		
Differentiation	n=7 Well	20 moderately	n= 1 poorly
pT Stage	T1 8 (27.6%)		T2 21 (65.6%)
LN +ve /LN	Mean 0.375, [0-1], SD= 0.20		
CRM	Clear 21 (87.5%)		Inconclusive 3 (12.5%)
LVI	Positive 1 (5.6%)		negative 17 (94.4%)
PNI	Positive 0 (0%)		Negative 20 (100%)

Table 19: Stage I Patient's Demographics and Pathology.

Outcomes for Stage I				
	Yes		No	
Recurrence	7		22	
	Local n=1	Distant n=6		
Mean Time Recurrence	11.5 months [3-25], SD=8.57			
Cancer Related Deaths	n=0			

Molecular Staging I				
KRAS	Mutant n=11 (47.8%)		Wild type n=12 (52.2%)	
BRAF				
MSI	MSI-H n=0 (0%)		Stable n=20 (100%)	
P16	Mean 16.85% [1-50] SD=14.40			
MGMT	Non-preserved n=1		n=15, Preserved	
MMR	Non-preserved 0 (0%)		Preserved 20 (100%)	
B-catenin	M 8 (53.3%)	M + focal N 4 (26.7%)	Mixed N+M 3 (20.0%)	N 0 (0%)

KRAS Status I	*Vs*. Feature			Total/*p* value
	No Recurrence	Local	Dis. Recurrence	
Wild type	11	0	1	12
Mutant	6	0	5	11 *p*=0.045

B-Catenin Status I	*Vs*. feature			Total/*p*-value
	No Recurrence	Local	Distant	
M	5	0	3	7
M + focal N	4	0	1	4
M + N	3	0	0	3 *p*=0.098

Table 20: Molecular biomarkers vs. outcomes for Stage I rectal cancer.

9 Discussion

Understanding the significance of molecular biomarkers for prognosis and treatment stratification remains an equivocal subject. Recent advances in molecular biology have greatly improved our knowledge of CRC, and significant progress has been made towards the identification of powerful prognostic as well as predictive biomarkers [78, 80, 119]. However, apart from a few well-studied genes or methylation products, identification of most of the current biomarkers remains controversial, which makes it challenging to implement them in clinical practice.

Looking through the most recent literature [73, 79], we found that most studies divide CRC carcinogenesis pathways into 3 distinct categories: CIN, MSI, and CpG island methylation (CIMP) pathway. The mechanism of these pathways is to try to elucidate CRC biology background by simplifying certain molecular events that share the same concept. However, despite all the efforts to distinguish certain molecular characteristics of each tumour and create a unique classification model [78, 80-82, 115, 119, 125, 137, 141, 167], there is still a significant knowledge gap to fill, which is primarily attributed to the complexity of colorectal carcinogenesis.

Currently, most studies divide biomarkers into prognostic and predictive categories. Prognostic biomarkers are those whose primary function is to delineate cancer-related survival, whilst predictive biomarkers refer to response to chemotherapy. For example, KRAS is a well-established predictive biomarker, as KRAS wild type is generally linked with a more favourable response to anti-EGFR chemotherapy [85]. On the other hand, BRAF V600E

mutation and MSI are gaining recognition in the literature as prognostic biomarkers, with the former indicating a poorer prognosis and the latter improved overall survival.

As discussed extensively in previous chapters, most of our work was based on a panel of biomarkers that was highlighted in the literature as the most influential in terms of its prognostic and predictive value [77, 78, 103, 104]. MSI has been extensively discussed in several papers, and its presence is linked with better overall survival, mucinous differentiation, and proximal tumour location [77, 78, 103, 126, 128, 133]. CpG island methylation (CIMP) is a promising family of biomarkers that are gaining more ground in CRC classification [75, 81, 82, 116]. CpG islands are generally part of the promoter region of several genes, and their abnormal methylation results in transcriptional silencing of either tumour suppressor or DNA repair genes [81, 82, 115, 127]. A classic example of CIMP is the abnormal methylation of the MLH1 gene promoter, which results in MSI and subsequently links CIMP-positive phenotypes with MSI-high. In our cohorts, we did a thorough study of β-catenin, p16, and MGMT methylation products, which were part of the biomarkers panel run by the Advanced Diagnostics Laboratory at King's College Hospital NHS Foundation Trust (KCH) [77, 104].

Despite the complexity in the understanding of molecular biomarkers, current advances in screening programs have made earlier diagnosis of CRC possible [206]. In that context, development of minimally invasive surgical techniques such as local excision is becoming more popular. Although local excision in the treatment of CRC is associated with reduced morbidity and mortality [41], the challenge of maintaining a similar recurrence rate compared to that of radical surgery remains a principal limitation for its application. Therefore, careful selection of patients is paramount for the achievement of optimal oncological outcomes. Transanal endoscopic microsurgery (TEMS) is one of the most favoured local excision techniques, initially introduced for the treatment of benign rectal tumours, for which it offers

the advantages of better technical and more complete excision in comparison to the older TAR technique [41, 104, 217-219].

In the context of the increasing rate of diagnosis of rectal cancer at an earlier stage, and given the current trend for tissue-preservation surgical techniques, we explored the significance of those biomarker panels in an early stage I rectal cancer cohort [104]. We also compared the significance of the same biomarkers in a cohort that underwent radical surgery for rectal cancer from the same specialist centre (KCH) in a similar period of time [77].

Furthermore, we performed a pilot study on 41 confirmed CRC cases selected from 1,500 consecutive referrals from the 2-week-wait pathway to figure out whether any of those biomarkers is linked with certain clinical or biochemical features at diagnosis [103]. This would help us delineate the difference between left and right cancer biology and identify those biomarkers that are more applicable to rectal cancer. For example, unexplained IDA was flagged as a worrying right-sided CRC symptom which was associated with the BRAF V600E mutation. Our findings are in keeping with the current literature [93, 113, 184] and raise the possibility of inclusion of BRAF biomarkers in screening tools in order to stratify potential risk for advanced cancer, as reflected by their relationship with IDA.

In a nutshell, we aimed to identify the prognostic significance as well as the predictive value of the most important biomarkers, based on the oncological outcomes of early cancer treatment with TEMS. However, along with TEMS, management of early-stage cancers involves neoadjuvant and/or adjuvant radiotherapy, which remain a cornerstone supplement either as downstaging (neoadjuvant) or as additional treatment (adjuvant). Therefore, the last chapter of this dissertation aims to determine the importance of neo+/-adjuvant radiotherapy in the oncological outcomes of TEMS and to delineate the possibility of a better treatment stratification protocol, along with biomarkers, in the context of an MDT approach [63, 220].

9.1 Kirsten rat sarcoma viral oncogene homolog (KRAS) in early rectal cancer (Stage I)

KRAS is a proto-oncogene involved in the cellular response to extracellular signals, and it is strongly associated with downregulation of mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase/v-akt murine thymoma viral oncogene (PI3K/AKT) pathways [69, 70, 132]. This means that surface tyrosine kinase receptors such as epidermal growth factor receptor (EGFR) are becoming resistant to inhibition [119]. However, there is a large family of chemotherapy agents consisting of anti-EGFR agents that target these receptors. Hence, KRAS mutant status is directly associated with chemotherapy resistance, which in clinical practice is used as a predictive biomarker [70, 76, 134-136].

KRAS mutations refer to a frequent alteration of G>A, a mutation most likely found in codon 12. Codons 12 and 13 in exon 2 constitute 90% of KRAS mutations [85], which has been the case with all our original cohorts [77, 103, 104]. In total, there have been 85 different mutations reported, many of which are pathway-specific [198]. Female gender is associated with KRAS mutant status tumours [85]. The Melbourne Collaborative Cohort Study concluded that KRAS mutations are associated with mucinous differentiation, and they appear in bimodal distribution along the proximal-distal axis, with the vast majority presenting proximally [85].

In the classic adenoma-carcinoma progression pathway, KRAS seems to appear early in the sequential neoplastic route. In 1988, Vogelstein et al. [221] stated that KRAS mutations appear after deregulation of the wingless-related integration site (WNT) pathway, most likely through APC mutation prior to TP53 gene inactivation. This theory has been accepted by recent studies [85] which delineated the distinct clinicopathological features that KRAS mutant tumours carry. In the same study, with one of the biggest original cohorts in the literature, KRAS mutant

status appears to affect 28% of the CRC specimens whilst generally the incidence fluctuates from 35−40%.

Nevertheless, the prognostic value of KRAS still remains equivocal. Although some researchers associate it with poorer prognosis [90], the vast majority doubt its prognostic value [85, 177]. In that context, we were one of the first to report that KRAS mutant status is associated with a higher recurrence rate in an early rectal cancer cohort following TEMS [104]. This is in keeping with the molecular basic that KRAS appears early in the carcinogenesis pathway [166]. Further, we introduced morphological features that could explain its prognostic value, including higher circumferential configuration ($p<0.0001$), larger tumour mass ($p=0.029$), as well as height of the specimen ($p=0.030$). Similarly, another team in the US supported the finding that codon 13 KRAS mutation can predict recurrence in patients undergoing hepatectomy for colorectal liver metastasis. [87-89]. A series of papers have described the relationship of KRAS codon 12 or 13 mutation status with the possibility for liver metastases of colorectal origin, highlighting the prognostic value of KRAS status. A recent meta-analysis confirmed the negative association of KRAS and overall survival, including poorer disease-free survival for patients undergoing liver resection for metastatic CRC. Another meta-analysis focusing on the prognostic value of KRAS using cell-free DNA concluded that KRAS affects CRC survival with a hazard ratio (HR) of 1.67 (95% CI 1.25–2.42, $p<0.01$) [91]. Finally, Rui et al. [222] in their meta-analysis link KRAS mutant status with worse overall survival.

Along with this novel finding, our original cohort of TEMS [104] revealed some more interesting associations. There seems to be a link between aberrant methylation of CpG islands, especially hyperexpression of membranous β-catenin with mutant KRAS status. This is confirmed by a previous study which states that 70% of KRAS mutant tumours are thought to have aberrant methylation profiles, including hyperexpression of p14, p15, and p16 [204]. This

finding is also supported by the fact that, as part of the same study, hyperexpression of p16 is associated with earlier recurrence, and hence this draws an indirect link with KRAS mutant status as well as other methylation products in the context of early rectal cancer.

To confirm our findings, we performed another original study in which we investigated the significance of the same panel of biomarkers. Focusing on KRAS status, we were gratified to confirm that there is still an association between early cancer specimens (Stage I) and distant disease recurrence when KRAS is mutant (n=4, p=0.045) [77]. Also, KRAS mutant status was associated with older age (69.62 vs. 62.27, p=0.005) and female gender (n=20, p=0.021) which is in keeping with reports in the current literature [85]. In the same study cohort, we did not find any further associations between KRAS status and distinct histopathological features, including LVI, the ratio of positive lymph nodes to lymph nodes, perineural invasion, and advanced stage of disease.

Similarly, there was no association between KRAS mutant status and LVI or disease stage in our first original cohort [103]. In this pilot study, we screened 1,446 consecutive 2WW referrals and confirmed 41 cancer cases whose clinical, biochemical, and histological presentation were evaluated against the molecular profile for the same biomarker panel. KRAS was not associated with advanced disease stage, and this study primarily focused on exploring the effect of BRAF V600E mutation in cases of unexplained IDA.

In conclusion, with our cohorts we delineated KRAS mutant status as a potential prognostic biomarker for early rectal cancer, since it was associated with a higher recurrence rate. This was supported by the fact that there is a link between aberrant methylation expression of p16 and membranous β-catenin with the classic codon 12 and 13 point mutations. It is important to note that KRAS is currently a broadly used biomarker across several cancer units in the UK, primarily as a predictive biomarker for response to chemotherapy. Recent advances in cancer screening have made it possible for a vast majority of CRC cases to be diagnosed at an earlier

stage, and for local excision techniques to be a potential option. On this background, our findings may be promising and KRAS could be used in combination with methylation products as an early-stage cancer prognostic biomarker.

9.2 v-raf murine sarcoma viral oncogene homolog B V600E mutation (BRAF V600E)

As previously discussed, the BRAF gene is involved in the RAS/MAPK intracellular signalling pathway [78]. Because it encodes a serine-threonine kinase that inhibits the RAS/MAPK pathway; therefore, any mutation can cause upregulation of the aforementioned pathway. The BRAF V600E mutation accounts for approximately 90% of BRAF mutations, and it is classically associated with MSI-high phenotype [93]. Most studies have associated BRAF V600E with proximal tumour location and in most cases, it is associated with CIMP-high phenotype and subsequently with MSI-high [80, 82, 119].

In their recent meta-analysis, Chen et al. [93] summarised all the clinicopathological features of BRAF-V600E based on those of 11,675 patients in 24 studies. Female gender (OR=1.71; 95% CI=1.42−2.07) was associated with BRAF V600E. Age above 60 years [OR=2.29; 95% CI=1.13−4.61) was another characteristic of this mutation and this was based on a sample of 2,982 patients. A very important aspect of BRAF mutation is its association with advanced disease stage at diagnosis (Stage III and above, OR=1.59; 95% CI=1.16−2.17). There is also an association with poor differentiation (OR=3.89; 95% CI=2.94–5.17), as well as with mucinous histology (OR=2.99; 95% CI=2.20–4.07) and proximal location (OR=4.85; 95% CI=3.59–6.56).

This same meta-analysis [93] confirms the association between BRAF V600E and MSI-high (OR=8.18; 95% CI=5.08–13.17) as well as CIMP-high (OR=16.44; 95% CI=6.72–40.21) that

many previous studies had already reported, as highlighted in our review [78]. With regard to KRAS status, there was a link with wt-KRAS and BRAF V600E, underscoring the probability that they derive from different pathways. In summary, this meta-analysis validated the association of BRAF mutant status with MSI and CIMP. Given the fact that 85% of sporadic CRC includes features of CIN and that the other 15% include the MSI-phenotype [223], this finding can be used as a guide to further delineate the way in which CRC follows distinct molecular pathways.

In our retrospective study [103], we tried to clarify the association between any of the current panels of biomarkers used by King's College Hospital with a clinical presentation of 41 confirmed cancer cases derived from a pool of 1,446 consecutive referrals from GPs. The main finding from this retrospective study was that BRAF V600E was associated with right-sided tumours and subsequent unexplained related iron-deficiency anaemia (IDA). BRAF mt tumours presented with a significantly lower Hb compared to those with BRAF V600E (89.0 vs. 123.5g/dl, p=0.009). Overall, we examined 26 patients with a colonic primary and 15 with a rectal primary. All the rectal cancer cases were found to be BRAF wild type, which is in keeping with the current trend in the literature. There was no significant association between presentation Hb levels and eventual disease staging ($p > 0.05$ for all associations). Patients with right-sided tumours were found to have significantly lower Hb levels compared to those with rectal tumours (98.6 vs. 122.3 g/dl), a fact that is well known in the literature.

One of the strengths of this pilot study was the comparison of patients' symptoms at presentation, including CIBH, iron-deficiency anaemia (IDA), rectal bleeding (RB), family history of bowel cancer, and presence of an abdominal mass, with the biomarker profile and the histopathological features, e.g., LVI. Apart from the association between BRAF V600E and significantly lower Hb, we did not find any further associations.

Summarising our findings and correlating them with the clinical practice, it is important to comment that although a lower Hb can be a feature of right-sided tumours and can be subsequently related to BRAF V600E, any case of unexplained IDA should be investigated and the possibility of advanced stage disease should be considered. This may affect the choice of treatment in cases of unexplained IDA, where BRAF status should be further examined. This corresponds to the current knowledge that BRAF is linked with advanced stage disease [93, 177, 179], and in our cohort, this was confirmed because all the BRAF V600E cases were T3b stage and above. However, there was no statistically significant association between BRAF V600E mutation and LVI, which could be due to missing data on the histopathological status of the specimens.

Another interesting feature of that study is the suggestion that BRAF should be included in the newly introduced Cologuard screening test [185], which is a multi-target DNA stool test based on the faecal immunochemical test (FIT). It is considered a noninvasive alternative to colonoscopy due to a sensitivity of 92.3%, which could be increased if BRAF status were included in this FIT.

With regard to the rest of our studies, both in the TEMS cohort as well as the rectal cancer cohort, BRAF V600E provided limited input in terms of a novel knowledge in the literature [77, 104]. This is primarily because BRAF-V600E is mostly found in proximal tumours, as discussed previously. All of our rectal specimens, both early and more advanced stage, were found to be BRAF wild type. The described relationship between BRAF V600E mutation and advanced disease stage could be due to the differing embryological origins and consequently the clinicopathological features of the right (midgut) and left (hindgut) colon [98].

9.3 Hypermethylation of CpG islands (CIMP) as a colorectal cancer biomarker

Methylation of CpG Islands (CIMP) is a fundamental aetiologic factor for cancer progression in molecular biology [81, 82, 116]. Aberrant hypermethylation can be classified further as global or regional. A classic example of regional hypermethylation is the aberrant methylation of unmethylated areas, most of which are CpG clusters [92, 224]. Tumour suppressor gene promoter or DNA repair gene promoter methylation occur in typical cases of CIMP aberrant methylation. Most studies have developed a classification model based on CIMP status [81, 116]. Generally, CIMP is defined as hypermethylation of CpG, which results in transcriptional silencing of the promoters of various tumour-suppressor and DNA genes [78]. A classic example of the latter is the silencing of the promoter of the mutS Homolog-1 (MLH-1) gene, which is a DNA repair gene. This is the pathway where CIMP and MSI meet, and hence the fact of their coexistence is fairly common in the literature. Simons et al. [82] suggest a classification model based on CIMP, MSI, and CIN. More specifically, according to the same study, MSI- and CIMP-positive tumours are proximally located, whereas CIMP-positive and CIN or CIN-only tumours are more distally located. Triple-negative tumours are those that are 89% BRAF V600E positive.

Prior to discussing our findings, we will focus on understanding the context and role of CpG as a mechanism in CRC both as a specific pathway, as well as in the complex context of the rest of the biomarkers. A recent meta-analysis [92] summarises the current evidence regarding CpG island methylation and its association with the rest of the molecular biomarkers. In this meta-analysis [92], 29 studies and 9,393 patients were involved. In the CIMP-high sub-type of CRC, there was a clear association between BRAF mutations (OR 34.87; 95% CI, 22.49–54.06) and MSI-high (OR 12.85, 95% CI, 8.84–18.68). KRAS mutations were less frequent (OR 0.47; 95% CI, 0.30–0.75) compared to CIMP negative. Also, the CIMP-high CRC subset shows a clear trend to older age at diagnosis (WMD 2.77; 95% CI, 1.15–4.38); mucinous histology (OR 3.81; 95% CI, 2.93–4.95); proximal location (OR 6.91; 95% CI, 5.17–9.23); and

poor differentiation (OR 4.22; 95% CI, 2.52–7.08). CIMP was associated with shorter survival (HR 1.73; 95% CI, 1.27–2.37), although no clear association was identified with tumour stage (OR 1.10; 95% CI, 0.82–1.46). Hence, according to those findings, CIMP can be considered as a prognostic marker which can potentially overlap with BRAF mutant status.

In our studies, we looked at the status of p16, β-catenin, MGMT, and MMR, all of which are methylation products that can be measured with IHC methods. In terms of p16, we measured the percentage of expression and correlated this with survival outcomes and clinicohistopathological features. B-catenin can be found localised either as membranous status, mixed membranous and nucleus, or nucleus. B-catenin status was further correlated with the same survival outcomes and clinicopathological outcomes. In the case of MGMT, we identified whether or not it was preserved and subsequently correlated it with the same outcome variables.

9.4 p16

P16 encodes a tumour-suppressor gene whose role is to bind with CD4/6 to stop interaction with cyclin D [225]. This prevents the cell progressing from G1 to S phase. Hypermethylation of p16 causes downregulation of the gene activity and hyperproliferation of cancer cells.

P16 seems to be a valid biomarker as 30−68% of sporadic CRC express hypermethylated p16 [75]. Many studies [224, 225] associate p16 protein upregulation in a stepwise fashion with colorectal adenoma and colorectal carcinoma. Al-Ahwal et al. [225] note in their results that p16 was significantly higher in CRC compared to in adenomas (p=0.033) and normal colonic mucosa (p=0.005). A recent systematic review on DNA hypermethylation [75] correlated the presence of p16 as an advanced stage feature, where persistent elevation may be associated with recurrence. This was one of the main findings from our TEMS cohort [104]. The p16 expression trend was associated with a shorter time-to-recurrence. More specifically, p16

expression >5% was associated with recurrence within the first 11.70 months. This could be a very important finding because p16 would serve as a biomarker for treatment stratification in early rectal cancer, and its expression trend could be a prognostic marker for future recurrence. However, no other association between p16 and other clinicohistopathological features was found. With regard to the rectal cancer cohort who underwent radical surgery +/- chemoradiotherapy [77], we found an increased trend of p16 expression along with membranous β-catenin compared to membranous/nucleus and nucleus (21.66 vs. 10.25 vs. 5.00, p=0.028). However, there was no association with any other biomarker or prognostic outcome. This could be justified by the fact that there were some missing data in the actual cohort, which made it harder to interpret the results.

Overall higher trends of p16 can be a promising indicator of prognosis, especially when combined with other already established biomarkers.

9.5 O^6 methylguanine-DNA methyltransferase (MGMT)

MGMT is a DNA repair protein which is thought to be involved early in the carcinogenesis pathway [226]. MGMT protein is coded at 10q 26 [200, 214], and it removes cytotoxic products from the O^6-guanine by transferring the alkyl group to an active cysteine, which is part of the MGMT sequence [216, 227]. Following this fundamental enzymatic reaction, the MGMT protein cannot be used again [215]. This implies its very significant role in the protection of the colorectal epithelium from various mutagenic agents throughout the replication process. According to a recent meta-analysis [226], MGMT is involved at an early stage in the carcinogenesis pathway, and its expression is altered even in the case of adenomas. The way expression is regulated is through methylation of the promoter of MGMT gene. The normal colorectal mucosa holds low levels of methylated MGMT [228, 229]. Previous studies have

compared MGMT methylation status across various stages of the carcinogenesis pathway from adenoma to carcinoma. Their important conclusion is that MGMT methylation exists even at the adenoma phase, which makes it a potentially useful biomarker for early-stage cancer [228, 230]. The outcome of the meta-analysis by Li et al. [226] was that MGMT protein expression precisely indicates its capacity to repair the DNA, and hence MGMT-preserved protein expression is a feature of the normal mucosa with good repairing potential. On the other hand, MGMT methylated status or loss of MGMT are poor prognostic markers. Loss of MGMT status is found in 30−40% of metastatic CRC [80, 116, 200, 215]. Although the role of MGMT is fairly well understood, there is still a long way to go in delineating the exact prognostic value of preserved vs. methylated status and reaching a consensus across researchers [80, 116, 200]. The Advanced Diagnostics Laboratory at King's College Hospital runs MGMT methylation status as part of its routine CRC molecular staging. Hence, as part of our studies we evaluated the status of MGMT against various clinicopathological parameters and survival outcomes, including recurrence.

In our first study [104], which involved early Stage I rectal cancer specimens of cases who underwent TEMS, MGMT was preserved in 91% (n=23) and non-preserved in 9% (n=2). MGMT non-preserved status was associated with advanced age (p=0.024 for age >80 years) and pT2 stage (n=2, p=0.030), which correlates with what currently exists in the literature. However, MGMT status was not associated with any recurrence outcome (p >0.05), and this can be attributed to the limitations of the small cohort.

In the second rectal cancer [77] cohort (2010-2014) who were treated with surgery +/- neo/adjuvant chemo/radiotherapy, MGMT was preserved in 63 cases (94%) and non-preserved in 4 cases (6%). Again, non-preserved MGMT status was associated with older age (83.50 vs. 66.53, p=0.005). Other than that, there was no other significant association noted. In the Stage I subgroup analysis of the same cohort, MGMT was available only in 16 cases, and in 1 case

it was non-preserved. Nevertheless, due to the size limitations we could not draw any further statistically significant associations.

In conclusion, MGMT can be perceived as a promising biomarker, especially for early rectal cancer, based on its molecular background as well as on the studies that have been published in various countries.

9.6 βeta-catenin

βeta-catenin is a fundamental pivotal molecule which is involved in oncogenic pathways and mainly in intracellular adhesion [205]. B-catenin is part of the Wnt-signaling pathway, which initially was thought to be regulated at the protein level; however, emerging data show that its regulation is at the gene promoter level [205]. To understand the role of β-catenin in CRC, we should focus on the "*liver CRC metastasis"* model. Initially, colorectal carcinomas appear to undergo a dedifferentiation process, and the actual result is a transition from epithelial to mesenchymal phenotype. This serves as a key mechanism for invasion and metastasis, known in the literature as epithelial-mesenchymal transition (EMT) [231-234]. The opposite phenomenon occurs in the inner parts of metastasis where cells are converting from mesenchymal to epithelial phenotypes, or in other words, re-differentiation. This process is known as mesenchymal-epithelial transition (MET) and implies that metastasis is a dynamic situation in which the tumour cells interact in their microenvironment via cytokines as well as matrix metalloproteinases (MMPs) and hypoxia. Coming back to the role of β-catenin, the dynamic EMT/MET transitions are thought to be regulated by β-catenin [231-234].

Localization of β-catenin in the cancer cell is linked with the development of cancer growth [235]. More specifically, nuclear β-catenin acts as a transcriptional activator of various genes—including the T-cell factor/lymphoid enhancer factor (TCF/LEF) family [74, 236]—and

therefore enhances tumour-promoting potential. Increased nuclear β-catenin is a feature of metastatic CRC and expresses the invasive potential of the tumour cells.

A study by Bandapalli et al. [205] supports this finding: 55% of their tumour specimens revealed elevated β-catenin mRNA, suggesting that β-catenin has a transcriptional regulation mechanism, which is a subsequent event arising from the interaction between the cancer cell and the tumour microenvironment. Based on that observation, they proposed that there should a dynamic transcription mechanism pattern, where transcriptional activation of the β-catenin promoter results in increased nuclear concentration of β-catenin and transcriptional activation of β-catenin-TCF-4. This mechanism promotes the EMT transition mechanism and therefore successful invasion, as described previously. The Wnt pathway has been studied in various other cancer models, including those for lung cancer [176].

In our early-cancer cohort who underwent TEMS [104], β-catenin was found to be membranous in 12 cases (48%); membranous and 30% nucleus in 7 cases (30%); membranous and 50% nucleus in 3 cases (10.3%); and nucleus in 3 cases (10.3%). All KRAS mutant tumours were associated with membranous β-catenin (n=8, p=0.009), whereas wild type KRAS were spread across various combinations of membranous/nucleus β-catenin. This may be attributed to the fact that early stage cancer has less potential for invasive spread and may follow different routes later on the carcinogenesis pathway. For example, nucleus β-catenin is a predominant finding of metastatic deposits of CRC in the liver [205]. In the case of early cancer, there is no clear timeframe when β-catenin transcription gets upregulated and the EMT pathway begins to work. Also, our study concluded that the KRAS mutant is linked with a higher local recurrence potential. However, although local recurrence itself is a poor prognostic marker, it does not represent a direct transition to metastasis, and there is still another branch of events that happen between recurrence and spread of disease. It would have been prudent to investigate the potential of distant recurrence and nucleus β-catenin. In that case, β-catenin

could have been a good predictor since distant recurrence can be an event which requires increased invasive potential.

Another limitation of this study is the small cohort size. This does not allow for generalisable results, and potentially makes it difficult to compare the early rectal cancer context with the already existing literature on β-catenin, which primarily refers to metastatic CRC. On a separate note, membranous β-catenin was associated with higher circumferential configuration of the tumour. Again, it is difficult to delineate the significance of this finding in the context of a small cohort of cases. However, a higher circumferential configuration level was associated with KRAS mutant status. Putting this together, we may assume that early cancer can be more complex to interpret, in the context of the different potential mechanisms of cancer progression. There was no other association of β-catenin status with survival or histopathological outcomes.

Hence, although it is clear that β-catenin plays a role in cancer invasion and progression, the stage at which altered localization can be a prognostic marker has not yet been identified, and further research focused on early cancer is essential.

Moving forward to our second cohort of rectal cancer cases [77], β-catenin was available in 63 cases (47% of cohort). B-catenin was membranous in 27 cases (42.9%); membranous + nucleus in 10 cases (15.9%); membranous with focal nucleus in 22 cases (34.9%); and nucleus in 4 cases (6.3%). Interestingly enough, membranous β-catenin was associated with a higher trend of overall recurrence in the early rectal cancer subgroup analysis, although this did not produce a statistically significant result (n=3, p=0.096). However, in the overall cohort there is a slight trend for membranous β-catenin to be associated with distant recurrence (n=6, p=0.092) although this again was not statistically significant. Furthermore, membranous β-catenin was associated with higher p16 expression trends when compared to mixed localization (membranous or nucleus) and nucleus (21.66 vs. 10.25 vs. 5.00, p= 0.028). Finally, there was

no association between β-catenin status and any histopathological features ($p > 0.05$ for all associations).

This slight deviation of our results compared to the overall literature can be attributed to 3 main factors. First, the limitations of a retrospective study with limited data can be a confounding factor that makes it difficult for us to interpret the results. Second, our cohort was composed exclusively of rectal cancer cases, which differ compared to what exists in the overall literature, where most studies discuss β-catenin in CRC in general. The third point is the fact that the assumption that nucleus β-catenin is associated with a higher invasive potential is based on metastatic CRC, where the EMT/MET pathway is primarily studied. Hence, examination of metastatic cells using IHC techniques may give a different picture. Our results derive from the original theatre biopsies that were processed at the Advanced Diagnostic Laboratory of King's College Hospital for completion of the pathology staging.

In conclusion, β-catenin is an important prognostic marker in CRC. Its biological background involves this protein in cell adhesion and invasion. Nucleic localization can indicate a high invasive potential in metastatic cohorts. Further research to evaluate its significance in early cancer prognosis is essential. Despite the limitations of our cohorts, it is clear that β-catenin is a significant prognostic marker which needs to be taken into consideration for further treatment stratification.

9.7 Microsatellite instability (MSI)

Microsatellites (MS) are short, repetitive DNA sequences which are prone to frame-shift type mutations as well as base-pair substitutions during replication [70, 78]. The role of microsatellites in colorectal carcinogenesis has been extensively discussed in the literature, and

the first systematic review on their prognostic value was published in 2005 [237], confirming a significantly better overall prognosis when MSI is present.

The DNA Mismatch Repair System (MMR) has been studied since the early 1990s, and it has been recognised as the primary cause of hereditary nonpolyposis colorectal cancer (HNPCC) syndrome[9]. The MMR system is primarily responsible for proofreading the replication sequence outcome and correcting and synthesising errors that arise from DNA replication [10]. MMR, therefore, has a DNA replication surveillance role and prevents recombination of non-identical sequences [10]. Although MMR has been extensively studied in the case of HNPCC, it is also responsible for around 15−25% of cases of sporadic cancer. The remaining 85% of sporadic CRC arises from CIN patterns, which primarily involve aneuploidy, allelic losses, and translocations [237].

The eukaryotic DNA mismatch repair system resembles the one found in E. Coli; hence there may be a revolutionary process in between these two [9]. The primary difference is that E. coli has 3 series of MMR proteins, including MutL homologs, MutS, and MutH. The eukaryotic DNA mismatch repair system resembles this model, with the exception of the endonuclease MutH [9]. There are 6 MutS homologs (MSH1−MSH6) and 4 MutL homologs (MLH1-3 and PMS1). Each homolog has unique functionality and serves in several aspects of DNA safeguard [9]. In Lynch syndrome, the MutL/S homologs involved are MLH1, MSH2, MSH6, and PMS2 [78]. Sporadic CRC is more likely to be associated with MLH1, and this is primarily through a mechanism of hypermethylation of CpG islands of its promoters, as described extensively in the previous paragraphs.

Failure of the MMR repair system can result in microsatellite disorders [78]. The tumour-suppressor genes are ideal targets for MSI-pattern mutations because they contain repeated hypermutable sequences that can contribute to MSI-related carcinogenesis [9]. Depending on the presence or absence of MSI phenotype, most studies divide CRC patterns into MSI-high or

MSI-low. Some authors divide MSI status into MSI-high, MSI-low, and MSS (stable), but in most instances, MSS and MSI-low fall into the same category [78].

With regard to the prognostic and predictive values of MSI in CRC, most of the studies conclude that MSI-high has a better prognostic value compared to that of MSI-low. This has been confirmed by a meta-analysis in 2005 [237] and has been extensively discussed and acknowledged [9, 78, 79, 125, 126, 170, 175]. However, its predictive value remains equivocal. Ribic et al. [178] state that MSI-H tumours are less likely to respond to fluorouracil-based adjuvant therapy. A recent meta-analysis [238] including 14 studies and 9,212 subjects showed that 5FU treatment for MSI-H tumours did not offer any statistically significant improvement in the overall survival [HR 0.66 (95% CI: 0.43, 1.03)]. However, the same meta-analysis showed overall improved survival in MSS cases who received 5FU treatment, both for disease-free survival [HR of 0.62 (95% CI: 0.54, 0.71)] and for overall survival [HR of 0.65 (95% CI: 0.54, 0.79)]. Hence, those results are in keeping with the fact that MSI holds a predictive value of response to 5FU, apart from its already-known prognostic value. Nevertheless, regarding MSI prognostic value, there have been 2 recent meta-analyses [239] [240] that doubt the prognostic significance of MSI status in stage II CRC. Gkekas et al. [239] state that MSI screening in stage II CRC cannot be routinely recommended as their meta-analysis concluded no overall benefit for disease-free survival or overall survival in general.

MSI has been part of several CRC molecular classification models [80-82, 119], and it has been associated with several features. To start with, MSI-high CRC is primarily proximal, poorly differentiated, and has a higher incidence of female gender. It also has mucinous differentiation, increased age of onset, and prominent nucleolus [69, 80-82, 127].

With regard to MSI association with other biomarkers, MSI-high is paired with the CpG methylator phenotype (CIMP) [93] as well as BRAF V600E mutation, as indicated by a recent meta-analysis [93]. The association with CIMP is via the mechanism of hypermethylation of

MLH 1 homolog, as described previously. With regard to CIN, this is considered to be a different family with distinct features which will be discussed later on this chapter. According to Rosty et al. [85], KRAS mutant status is associated with more MSS ($p<0.001$, n=36). In contrary from 92 MSI-high cases, 83 were KRAS wild type ($p<0.001$).

With regard to our findings, in both our cohorts who received radical surgery [77] or TEMS [104], MSI status played a limited role because both series were related to distally located specimens (rectal). In the TEMS cohort, our specimens were microsatellite stable, which corresponds with other reports in the current literature; therefore, no further conclusions were drawn. In the second cohort of patients who received radical surgery +/- neo/adjuvant chemo/radiotherapy, only 1 case was MSI-High and 95 were MSS. Regarding associations, it was noted that MSI-H and overall preserved-MMR were associated with positive perineural invasion ($p=0.021$); however, this has limited value given the fact that it refers to only 1 case. On the subgroup Stage I analysis, all the cases demonstrated microsatellite stable phenotype and preserved MMR.

In summary, MSI is a particular molecular pathway which is responsible for 15−22% of CRC cases [9, 119] because of its effect on the DNA repair system. Predominantly, MSI is a feature of proximal tumours, and its prognostic value seems to be accepted by the vast majority of the studies published in the literature. MSI-H is a favourable indicator of prognosis, and the absence of MMR defects is deemed as a poor prognostic feature. All but 1 of our specimens were MSS, which conforms to the current trend of the literature; therefore our contribution to novel knowledge on this particular biomarker was limited. MSI is known to be linked with BRAF V600E mutation, and in most cases it appears to coexist with wild type KRAS, although there have been many studies that link MSI-H with KRAS mutant. Finally, MSI is strongly related to CIMP-high, as the latter explains the molecular mechanism by which the MLH1 gene is affected in sporadic cancer.

9.8 Chromosomal instability patterns (CIN) – the hallmark of colorectal cancer

CIN refers to a large proportion of colorectal tumorigenesis, and this is considered to be around 85% [69, 80, 82, 130, 131, 169, 241]. The biological definition of CIN is any chromosomal losses or gains that result in aneuploidy or polyploidy, respectively [78]. A recent narrative review summarises all the evidence acquired during the last decade [241]. The background behind CIN is based on the somatic-copy-number-alterations (SCNA) that cancer cells display. SCNA includes any focal events that cause segmental instability or general aneuploidy. As a result, around 70% of colorectal tumours display significant internal heterogeneity and deviate from diploid karyotypes, and this phenomenon is explained by CIN mechanisms [241-243].

Many studies consider CIN as a distinct mechanism of carcinogenesis [80, 82, 169], and tumours that do not encompass this mechanism are often classified as MSI-pattern tumours. As previously discussed, MSI involves all DNA repair mechanism failure, which includes mutations to DNA polymerase-ε and/or DNA polymerase-δ catalytic subunit 1 [241]. The MSI mechanism itself may cause the maximum instability that a cell can sustain before it drives to cell death.

Understanding of this molecular mechanism includes the fundamental assumption that CIN is the reason for temporal and spatial diversification of tumour subclones via intratumour heterogeneity through SCNAs [241]. Also, CIN allows cancer cells to diversify their phenotype via segmental or whole-chromosome aneuploidy, and hence tumour cells can try different complex genetic makeups before they reach lethality [241]. On the other hand, this means that CIN aneuploidy can potentially concentrate a significant amount of DNA mutations, which convert the cancer cell to non-sustainable and cause tumour suppression. However, any chromosomal alteration leads to a variation in tumour cell potentials and therefore a poor predictive outcome of cancer treatments, as the cell may be resistant to the chemotherapy

agents [241]. CIN influences all immune-checkpoint inhibition, and a deeper understanding of this process will be a key factor in optimising cancer treatment in the future [241].

In studies that delineate the CIN macroscopic features, CIN-positive tumours tend to have a poor prognosis and tend to be characterised as well or moderately differentiated [80]. Simons et al. [82] noted that CIN-only tumours occur slightly more frequently in males (54.7%) and 76.5% are at least Stage II when diagnosed. A study by Domingo et al. [80] makes the fundamental assumption that CIN is completely different from MSI cancer pathways. In our study, we have briefly explained the potential incompetence of the cell to accumulate both pathways. However, CIN+ tumours can be associated with TP53-mutant cancers to the CIN+ group. Domingo et al. did not find any negative association between KRAS or PIK3CA and CIN, and hence they divided the CIN group into KRAS/PIK3CA mutants and KRAS/PIK3CA wild type. Given the fact that TP53 has an inverse relationship with the KRAS pathway, they added the TP53 mutant to the KRAS wild type group and the TP53 wild type to the KRAS mutant group.

A study by Simons et al. [82] describing 509 CRC tumours attempted to delineate the distinct types of CRC carcinogenesis. According to this study, 58.2% of the tumours followed the CIN-only pathway and 13.4% adhered with CIN and CIMP. Increased poor survival rate was noted for CIN-only tumours, CIMP and CIN, triple-negative, and CIMP-only tumours. The maximum heterogeneity in terms of tumour groups was noted in CIMP, where there is a good mix of CIN (13.4%), MSI (12.6%), and CIMP-only (5.3%) cancers.

In their meta-analysis, Walther et al. [242] conclude that CIN tumours have a worse prognosis compared to the other CRC subtypes. Exploring the way Walther et al. synthetised the evidence; 63 studies were included and 10,126 CRC cases were analysed. With regard to CIN+ subtype prognosis, a worse prognosis was reported for 54 of the 63 studies. The overall HR associated with CIN was 1.45 (95% CI 1.35−1.55, $p<0.001$. To emphasise the impact of CIN

on the prognosis itself, Walther et al. did a subgroup analysis comparing patients who had not received any treatment or studies before 1987 with non-metastatic cases who had rarely received any chemotherapy. The overall HR in this case was 1.66 (95% CI 1.41−1.95, p<0.001; Q=17.34, I2=36.6%, p=0.098), which shows robust evidence of poorer prognosis in CIN subtypes [242].

Another really important conclusion from the same meta-analysis is that there was no robust conclusion about the predictive (response to chemotherapy) value of CIN for 5-FU-based agents.

In conclusion, CIN is a fundamental biomarker in CRC and a hallmark of oncology in general. Although we did not focus on CIN directly in our original cohorts, all our results were compared against the literature trends for the current classification models.

9.9 Comparison of various clinicohistopathological data with the biomarkers panel (KRAS, BRAF, β-catenin, p16, MGMT, MMR/MSI)

Although we have extensively discussed most of the biomarkers that we studied during our cohort analyses, it is interesting to see the impact of our biomarkers' status on clinicohistopathological outcomes in a holistic way. In the first cohort of patients who received TEMS [104], our main biomarkers were KRAS, β-catenin, and p16. BRAF was wild type in all cases, so we did not draw any further conclusions. Similarly, all the specimens had MMR-preserved and only 2 had MGMT non-preserved. MGMT non-preserved was associated with pT2 stage, although this can be attributed to sample limitations since it refers to only 2 cases (p=0.030). Therefore, there was no association between differentiation, dysplasia, tumour location, volume, or circumference, or excision margins with MGMT or MMR or p16 (p >0.05). Evidence across the literature is limited on these associations. A study by Chen et al.

[155] was one of the first to correlate clinicopathological data with methylation patterns and especially p16. More specifically, Chen et al. [155] associated the status of p16 promoter methylation with lymph node metastasis (p=0.002), histologic grade (p<0.001), tumour size (p=0.041) and location (p<0.001) as well as with stage of disease [TNM, (p=0.06)]. This is a promising finding that associates known histopathological parameters with the molecular background, from which significant conclusions can be drawn.

Back to our study, the most important findings were based on KRAS status. The context of our cohort was 29 early rectal cancer cases (pT1 or pT2–Stage I) that received local excision treatment and only 3 cases additional neoadjuvant radiotherapy. The main associations between KRAS mutant status and histological features involving greater tumour circumference (30−80 vs. 20−50%, p=0.000), pT stage, MRI pT stage, or tumour mass (cm^3) were not associated with KRAS mutant status. However, the tumour's mass (cm^3) was higher in the case of mutant KRAS (159.250 vs. 17940, p=0.073), although this was not statically significant.

With regard to the pilot study which consisted of 41 confirmed CRC cases [103] (any location), there was no statistically significant association between BRAF V600E mutation and LVI, although LVI positive was associated with lower Hb (109 vs. 115.1g/dl). BRAF V600E mutation was found in right-sided tumours with advanced disease stage (T3b and above), which is the case described in the latest meta-analysis [93]. With regard to KRAS status, there was no evidence of any association between mutant (codon 12 or 13) status and disease stage as p>0.05 for any association. Currently, there is no clear evidence in the literature of any association of KRAS status and disease stage [85].

Finally, in the rectal cancer cohort who received neo/adjuvant chemo/radiotherapy +/- radical surgery [77], there was no clear association between KRAS mutant status and positive lymph nodes (LN) or ratio of positive lymph nodes/lymph nodes (+ve LN/LN). The latter (+ve LN/LN) was not associated directly with any of the biomarkers studies (KRAS, β-catenin, p16,

BRAF, MMR) as $p>0.05$ for any relationship. This study did not reveal any new associations between our biomarkers and the histopathological outcomes. There have been studies in the literature which associate histopathological features directly with KRAS, BRAF, p16, MGMT, or MMR status, as described previously. However, there is still very limited evidence on the relationship of those biomarkers with perineural invasion (PNI), LVI, LN, or +ve LN/LN, and this is what we tried to delineate in this cohort. Further studies with larger cohorts may help in shedding light on the hidden link in the sequence of the events between molecular events and histopathological outcomes. A promising project towards this direction will be the 100K genome project, which will allow prospective gene mapping and a large, promising cohort of analysed cancer specimens across the UK [238, 244].

9.10 Conclusions

As we are moving towards an era when screening and earlier diagnosis of CRC will have been achieved, understanding this particular cancer field will improve long-term survival outcomes and decrease morbidity. Furthermore, in the context of evolving surgical techniques and newer technology becoming available, local excision techniques have been greatly improved in the last 2 decades, allowing a "tissue-sparing" approach to be possible. There has also been rapid progression in oncology, and new chemotherapy or radiotherapy adjuncts and techniques are now available. In that context of rapidly evolving science, a multidisciplinary approach to rectal cancer becomes essential for optimal results. However, given the complexity of the cancer biology, specific and sensitive prognostic and predictive biomarkers in early rectal cancer become a real challenge. Although there are extensive studies on advanced cancer and the relevant biomarkers, there is still a long way to go in delineating the significance of the existing biomarkers in the case of early cancer stages. This will allow optimal stratification of

treatment and reduce recurrence risks, which are definitely higher in the case of local tissue−sparing techniques.

One of our main findings is that KRAS mutant (codon 12 or 13) may be an important prognostic biomarker for early rectal cancer. This finding is something new, and definitely larger prospective studies are needed to evaluate how it can be useful and applied to clinical practice. KRAS is routinely tested in most of the cancer units in the UK for cases of positive histology. The question is whether early Stage I rectal cancers with mutant KRAS status follow a more aggressive pathway and whether they tend to reoccur either locally or distantly. Surprisingly enough, a similar finding came from the second cohort when we focused our analysis on early rectal cancer (stage I). Therefore, there is ground for a research hypothesis and a new, larger prospective study to take place in the future.

Undoubtedly, aberrant methylation is one of the most promising hallmarks in the understanding of cancer biology. More and more sporadic cancers are explained by hypermethylation of several promoter regions of CpG islands, which affects various tumour suppressors and oncogenes. In our studies, β-catenin and p16 seem to be involved, and their expression deviates from normal. This underscores the fact that methylated protein products can be useful and important biomarkers even at the earliest stages. Although there are a few meta-analyses on this subject, a deeper understanding is required to reach robust explanations of their role. We found that a higher p16 expression trend is associated with higher chances of early recurrence after local excision, which is partially confirmed by the current trends in the literature. B-catenin expression can shift from membranous to nucleus; however, the time point at which this phenomenon occurs remains unclear. More recent articles state that this is the case early on in the disease process, and therefore attention to this biomarker must be given even for Stage I cancers. Our cohorts of early-stage tumours revealed that a predominantly membranous β-catenin with an element of nucleus holds prognostic value. This may represent the beginning

of the process, and nucleus β-catenin is evolving as the predominant localization in later phases of carcinogenesis.

Finally, we suggested that unexplained IDA in the case of suspected cancer can be associated with BRAF V600E and right-sided advanced cancer. As we are shifting into an era of non-invasive CRC screening in which more DNA stool tests are becoming available, BRAF V600E would be a useful suggestion for inclusion in those cases.

9.11 Limitations

However, we recognise the limitations in our studies; most of the limitations have been discussed in each chapter separately. In general, the size of the 3 original cohorts can be considered as small (cohort 1: 41 specimens, TEMS cohort 2: 29 specimens, Rectal cancer cohort 135 specimens); nevertheless, most of TEMS original series reported in the literature are limited in terms of number of specimens.

Also, some of the studies incorporate retrospective collection. To optimize accuracy of data, most of the cases, especially in the TEMS cohort, were discussed both with the senior surgeon who performed the procedures (SP), as well as with the consultant histopathologist who interpreted the original histopathology. As this thesis was based on a single tertiary institution, we focused on samples from the local population. This can be a question for external validity, however, the representativeness of our population is adequate, given the fact that KCH is an international institution, and the geographic region cover a widely diverse population. Finally, with regards to statistical methodology, we focused on univariate inferential statistics as the sample size did not allow multivariate modelling. However, this was interpreted in the context of a deep understanding of the background (molecular biomarkers), which ensured that all our conclusions were drawn appropriately.

There were also some missing data in terms of molecular biomarkers, which unfortunately was attributed to cost implications. However, in most cases the data were available for analysis (either de novo lab-based work analysis, or some pre-analyzed biomarkers in the last cohort), and on top of that, the quality was extremely high, as all the results were double validated by JM who is an experienced senior scientist (Director of the Advanced Diagnostics Lab).

9.12 Future work

As in every other study in molecular oncology, this piece of work generates several and valuable questions. Further research is vital to delineate the significance of current biomarkers in early cancer cohorts. Also, identification of new biomarkers remains a hot topic in oncology, which may benefit from emerging evidence from miRNA molecular biology or metabolomics. Combining results from multiple centres, or equally retrospective synthetising of existing evidence using advanced statistics (individual Patients' Data – iPD meta-analysis) will help in developing an ultimate prediction model in which cancer treatment will be personalised to optimise survival, minimise morbidity, and improve patients' experience. Finally, as previously discussed, artificial intelligence and "big data" research methodology is rapidly evolving, allowing more questions to be answers via using advanced technology.

We do not know how far this may lead; however, a rapidly evolving multidisciplinary environment seems fairly promising for the near future.

10 Bibliography

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